Selection, production and characterisation of a biological indicator organism for low temperature steam and formaldehyde (LTSF) sterilization

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SELECTION, PRODUCTION AND CHARACTERISATION OF A BIOLOGICAL INDICATOR ORGANISM FOR LOW TEMPERATURE STEAM AND FORMALDEHYDE (LTSF) STERILIZATION

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Thesis

This research was carried out in the School of Pharmacy and Pharmacology of the University of Bath under the supervision of Dr C.J. Soper, B.Pharm., MSc., PhD., MRPharmS. and Dr D.J.G. Davies, MSc., PhD., FRPharmS.

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SUMMARY

This thesis reports on the search for a suitable Biological Indicator organism for use in monitoring the lethality of low temperature steam and formaldehyde (LTSF) sterilization.

The Introduction defines sterilization and includes a summary of the kinetics of sterilization processes. It also reviews several sterilization methods and their validation. The use of Biological Indicators both for validation and routine monitoring of lethality is discussed. Current theories of heat, chemical and radiation resistance in bacterial spores are also outlined. The physical properties, history as a bactericide and safety aspects of formaldehyde are also described.

The first experimental chapter describes the screening of 16 strains of *Bacillus stearothermophilus*, sporulated on various chemically defined media, for suitability as a Biological Indicator organism for LTSF sterilization. A possible candidate organism for development into a Biological Indicator is suggested.

Chapter 4 describes the modification of a commercial LTSF sterilizer into a suitable test apparatus to allow exposure of bacterial spores to defined LTSF conditions. This apparatus facilitated the controlled variation temperature and formaldehyde concentration to enable the effect of these parameters to be assessed.

Experiments reported in Chapter 5 describe the use of the test apparatus to characterise the resistance of the candidate organism, chosen in Chapter 3, to defined LTSF conditions. In particular the effect of changes in temperature and formaldehyde concentration on the resistance of the organism was investigated.

Chapter 6 reports on investigations into the effect of heat treatment of spores, which have previously been exposed to LTSF, prior to them being plated onto a recovery medium. The implications of the results presented in this chapter are discussed in terms of the mechanism of LTSF lethality and its future as a sterilizing process.

Chapter 8 is a concluding discussion of the data in relation to current knowledge. Suggestions for the direction of future studies are also included.

CONTENTS

ORIGIN AND SCOPE

It is necessary that surgical instruments and other medical items should be sterile before being used for intrusive medical operations. The established method of choice for sterilization of medical items is high temperature steam autoclaving. This method was chosen for its reliability and ease of monitoring, due to the process being precisely defined in physical terms. However, with the increasing use of heat sensitive medical apparatus, for example those made of thermolabile plastics or incorporating sophisticated microelectronics or optics, the search for an alternative to high temperature steam autoclaving has become increasingly important.

Many alternative sterilization processes have been proposed and tried, including ethylene oxide, ozone, U.V. radiation and gamma irradiation. All of these methods have major faults ranging from extreme toxity and hazard for ethylene oxide to detrimental effects on items with gamma irradiation. The most promising alternative proposed is the use of sub-atmospheric steam combined with gaseous formaldehyde, known as Low Temperature Steam and Formaldehyde (LTSF). Unfortunately simple physical measurement of factors such as temperature and pressure will not suffice to monitor the lethality of this process, and therefore Biological Indicators must be used. The Biological Indicators which are currently commercially available were not specifically designed for monitoring LTSF sterilization, and therefore their responses vary widely.

This investigation was instigated for several purposes. Firstly to select a suitable organism for use as a Biological Indicator for the LTSF process. Secondly, to develop a suitable apparatus that would allow the exposure of organisms to standard LTSF conditions. Finally an investigation into the responses of a candidate organism for a Biological Indicator to various LTSF conditions, allowing the

effect of various parameters on the lethality of the process to be demonstrated.

CHAPTER 1

INTRODUCTION

1.1 STERILIZATION

Sterility is defined as the absence of all viable life forms. In the healthcare industries this relates especially to micro-organisms. This term is an absolute one, and hence there can be no degrees of sterility. The process of sterilization is defined as the destruction or removal of all viable life forms in or on a product/object to achieve sterility. This includes even the most resistant bacterial spores, and hence is distinct from the process of disinfection, which is defined as the destruction of yeasts, moulds, vegetative micro-organisms and most viruses but not resistant bacterial spores. Sterilization may be carried out for a number of reasons and by a variety of methods. The reasons could be to allow safe use of medical products and instruments, to prolong shelf life of foodstuffs and medicines or to ensure that an experiment or process is carried out only with a desired micro-organism. Sterilization usually involves the exposure of the product/object to inimical physical or chemical agents for a predetermined length of time. The agents commonly used include heat (in the presence or absence of water), ionizing radiation or inactivating chemicals in liquid or gaseous form, usually together with heat. In the case of liquid products and gases, sterilization by filtration may be a possible method.for the removal of viable micro-organisms (Hardwidge *et al*, 1984). The choice of agent will be decided on the basis of several factors. These include the expense, practicality and the amount of throughput a process will require. Probably the most important reason for choice of process however is what conditions the product/object can tolerate without deleterious effects.

There are two major problems associated with the achievement of

sterility. The first of these is that there is no known method of demonstrating that sterility has been achieved. Methods such as the British Pharmacopoeia test for sterility rely upon the recovery of any viable micro-organisms present using culture media. Viable micro-organisms are detected by the formation of colonies on solid media, or by causing turbidity in liquid media. These positive results rely upon every single remaining viable micro-organism replicating through many generations to be visible by these methods. An organism which cannot replicate, or can do so only through several generations would be considered dead by this criterion. As organisms which have survived a sterilization process are often damaged, they may have specific nutritional and environmental needs to recover and repair the damage. These needs will vary both within and between species, according to the type of damage suffered. This means that an optimal medium and recovery conditions for all micro-organisms, (i.e. media and conditions which would recover all micro-organisms which still live after a sterilization process), is an impossibility, and therefore absence of viable micro-organisms can never be demonstrated with complete confidence.

The second problem associated with the achievement of sterility is that micro-organisms exposed to a lethal process do not all die at the same instant. To a first approximation, the number of viable micro-organisms decreases exponentially with the time of exposure to a lethal agent. This means that the number of viable micro-organisms will only reach zero after an infinite exposure time. Although there is always a finite probability of a micro-organism surviving a sterilization process, this probability can be reduced to an acceptably low level by proper design of the process. Therefore, in the healthcare industries a probability, or sterility assurance

level (SAL), of 1 in 1,000,000 (10^{-6}) units being unsterile is considered to be a practical definition of sterility (Pflug and Smith, 1988).

1.2 KINETICS OF MICROBIAL INACTIVATION.

A number of mathematical terms are used to express the resistance and death of micro-organisms. These terms are generally based on the assumption of death occuring exponentially with exposure time by first order kinetics.

1.2.1 Inactivation Rate Constant (k)

When a homogeneous population of micro-organisms is exposed to lethal conditions the proportion of viable organisms decreases in a regular fashion. It is thought that to a first approximation the rate of decrease in viable organisms is directly proportional to the number of viable organisms present at any instant of time. This means that a constant fraction of the viable population is inactivated for each instant of exposure time to the lethal conditions. This can be described mathematically by first-order kinetics as

$$N_t = N_o\, e^{-kt}$$

where N_t is the number of viable organisms after an exposure time of t, N_o is the number of viable organisms at time zero (the bioburden), t is the exposure time and k the inactivation rate constant. If the fraction of surviving organisms (N_t/N_o) is plotted on a logarithmic ordinate against exposure time on a linear abscissa the resulting plot is known as a survivor curve and has a negative slope of -

k/2.303 from which the inactivation rate constant can be calculated.

1.2.2 Arrhenius relationship

With first order inactivation kinetics, the inactivation rate constant, k, can be related to the absolute temperature of inactivation, T, by

$$k = Ae^{-Ea/RT}$$

where k = inactivation rate constant (min^{-1}); A = frequency factor ($time^{-1}$);

E_a = activation energy of bacterial death; R = universal gas constant; T = absolute temperature (K)

Alternatively this can be expressed in the logarithmic form :

$$\log k = \log A - E_a / 2.303\ RT$$

An Arrhenius plot is obtained by plotting k on a log scale against the reciprocal of the absolute temperature 1/T on a linear scale. This produces a linear plot with slope Ea/2.303. This allows the activation energy, E_a, of the lethal process to be calculated.

1.2.3 Decimal Reduction Time (D-value)

This is often used by microbiologists to express the resistance of a micro-organism to a lethal process, and is defined as the exposure time required to inactivate 90% of the microbial population. On a survivor curve plot, this is equivalent to the exposure time needed to obtain a one log cycle reduction in survivors. This value can be used to compare the resistances of different bacteria to an inactivating process. This value can be expressed mathematically as,

$$D = t / (\log N_o - \log N_t)$$

where N_o = initial viable count at t = 0, N_t =viable count at time t and t = exposure time.

The relationship between the D-value and k for a first-order exponential response is given by

$$D = 2.303/k$$

Both D and k values are specific to the particular conditions of the inactivation, and hence these conditions must be stated. In practice, the temperature of inactivation at which the D-value was obtained is shown as a subscript. Therefore the D-value for inactivation of an organism at 121°C would be D_{121}. D-values are only normally used to compare the resistances of organisms which exhibit linear survivor curves. It is possible to estimate D-values of non-linear survivor curves from their linear portions or from a straight line obtained by linear regression, but they do not represent the resistance of that organism fully. A more representative value for such composite comparisons is the T_3 value.

1.2.4 T_3 value

The T_3 value is defined as the exposure time required for a three log cycle decrease in the number of survivors on a survivor curve plot. This is used particularly for the comparison of non-linear survivor curves as it represents an average measure of the rate of inactivation over this time.

1.2.5 Inactivation Factor (IF)

This is defined as the number of log reductions on a survivor curve plot for a given sterilization process. This is used as a measure of the probability of an unsterile unit occuring in a

sterilization process. The IF is dependant on the D-value of the organism for which it is calculated, and is given by :

$$IF = 10^{t/D}$$

where D = D-value and t = exposure time

If an item was assumed to have an initial bioburden of 1×10^6 organisms, and was sterilized by a process with an IF of 10^{12}, then the chance of obtaining an unsterile item is 1 in a million (or 10^{-6}). This example is illustrated in Figure 1.1.

The British Pharmacopoeia (1988) recommends sterilizing at 121°C for 15 minutes. Using the biological monitor, *B.stearothermophilus* NCTC 10003, as recommended in this volume, which has a D-value of 1.5 minutes, the IF for this process would be 10^{10}.

1.2.6 Z-value

The Z-value is defined as the increase in temperature required to reduce the D-value of an organism by one log cycle on a plot of $\log_{10}$ D-value against temperature. This value is directly proportional to the resistance of the organism, and is a characteristic of that organism for the particular lethal process for which it was calculated.

1.2.7 F-value

The F-value is a 'unit of lethality', and is defined as the total process lethality. This is used to equate the inactivation obtained at any temperature to the time required at a particular reference temperature and reference organism to produce the same lethality (Soper, 1988). This is used to minimise sterilization times by taking into account the heating up and cooling down times of a

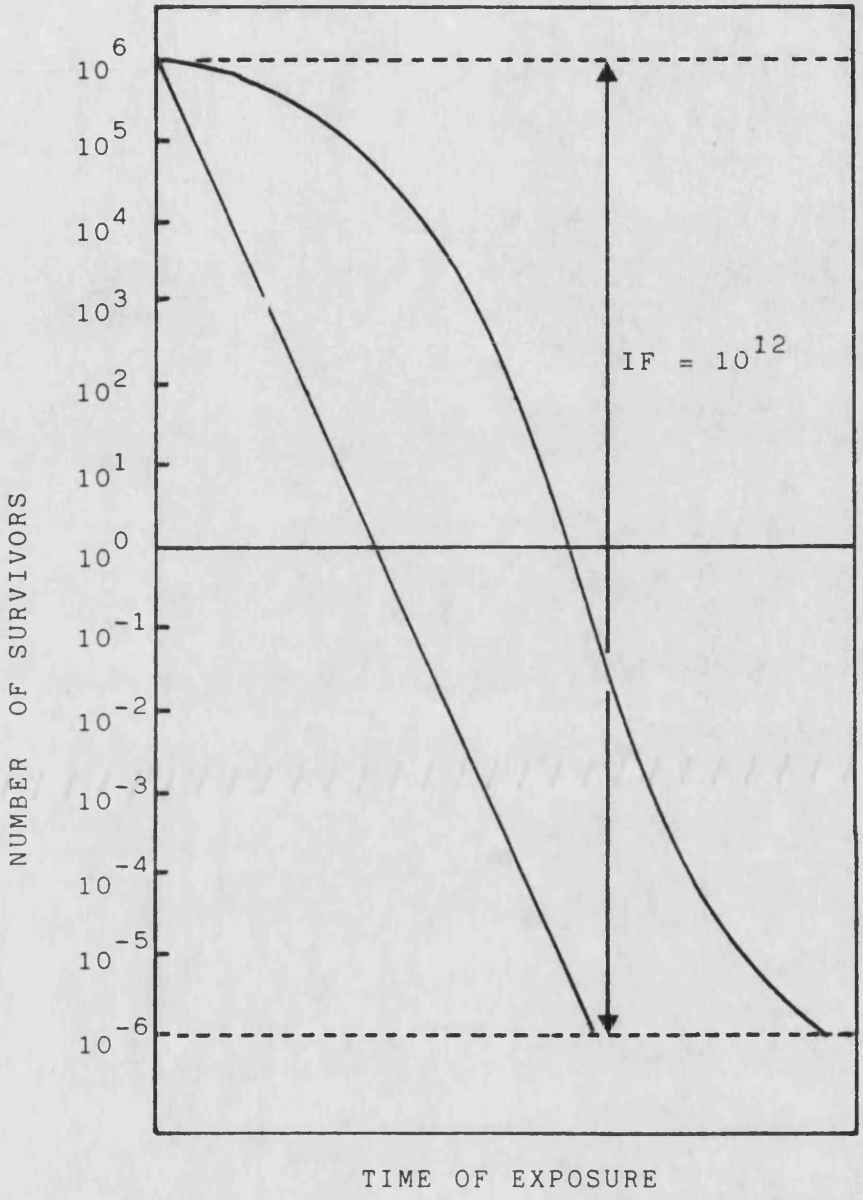


Figure 1.1 Log-linear and non-log-linear survivor Curves of Numbers of Survivors on a Log Scale Against Time, to Demonstrate IF for Time t. Modified from Soper, 1988

sterilization cycle, and converting this to equivalent time at the sterilizing temperature.

1.2.8 F_0-value

The F-value compares lethal effects of different temperatures and is thus dependant on the Z-value of the reference organism. Spores of *B. stearothermophilus*, having a Z-value of 10°C are therefore often used as a standard organism at a reference temperature of 121°C. Under these conditions the F-value is known as the F_0-value. A process with an F_0-value of 10 therefore has the same lethality on these spores as heating them at 121°C for 10 minutes.

The British Pharmacopoeia (1988) considers sterilization by autoclave satisfactory when it delivers an F_0-value of 8 to every container in the load.

1.3 STERILIZATION OF THERMO-LABILE MATERIALS.

There is an increasing use of heat-labile equipment and materials in modern hospitals, that is equipment that is incapable of withstanding the high temperatures used in steam (121°C-134°C) or dry heat (150-180°C) sterilization. These include equipment constructed of plastics and rubber material and electrical equipment. The use of these has meant that the search for a method of low temperature sterilization which is microbiologically reliable, and does not leave tissue irritants on the materials, has become more urgent. Glutaraldehyde is a liquid sterilant that is often used to sterilize endoscopes, and though proven effective against various organisms

(Collins, 1986 a,b), requires 3 hour immersion, is potentially toxic and recontamination may occur during the rinsing phase (Deverill and Cripps, 1981) and hence is not a good choice. Three methods appear to fulfill the necessary criteria mentioned above, at least in part, for such sterilization. These methods are gamma irradiation, ethylene oxide (EtO), and low temperature steam and formaldehyde sterilization (LTSF). Gamma irradiation is efficient and reliable and effective against bacterial spores (Borick and Fogarty, 1967), but its drawbacks are that it is generally not available locally and is therefore time consuming and expensive. Another drawback is that gamma irradiation has been demonstrated to have detrimental effects on some plastics including reduction in molecular weight and increase in cross-linking leading to loss of flexibility and increase in brittleness (Woolston, 1990). Ethylene oxide, though used widely in hospitals and industry, leaves toxic residues in and on the object sterilized. It requires a long degassing period to remove these residues thus reducing the turnover. An alternative to these methods is LTSF sterilization. However, for LTSF to be considered a reliable routine sterilization technique, it would be necessary to be able to monitor the effectiveness of the process with each load as can be done with heat sterilization methods. With processes such as high pressure steam sterilization, the variables temperature and time need to be monitored to assess the effectiveness of a cycle. These can readily be monitored using reliable physical methods. With a process such as LTSF, temperature, relative humidity, formaldehyde concentration, distribution and penetration into the load are all important factors affecting success of sterilization. It is not currently practicable to validate and routinely monitor these factors by physical methods, therefore alternatives such as biological

methods must be used.

1.4 VALIDATION OF STERILIZATION PROCESSES.

To validate the efficacy of a sterilization process, the sterility of the items processed must be assessed. There are two ways of assessing the effectiveness of sterilization, the first being that a statistically significant number of items are tested for sterility. The disadvantage of this is that sterility testing is destructive, prone to false positive results and items tested cannot be used afterwards. Finally no matter how large the size of the sample, sterility could not be guaranteed except by testing all items. This is due to the inherent problems in taking a representative sample (Caputo *et al*, 1980). The second method is to monitor the effectiveness of the sterilization process by physical, chemical or biological means, or a combination of these. In the design of a sterilization process, it is usual to select a reference organism, one considered likely to be more resistant to the process than the most resistant contaminating organisms. It is necessary to have an idea of the types of contaminating organisms in these cases. Bacterial spores are usually used as they are considered among the most resistant of all living organisms. If these spores are then attached to a carrier system and calibrated it is known as a Biological Indicator (BI). These despite their inherent variability, have been shown to be reasonably accurate and reproducible for use in the validation and routine monitoring of sterilization processes, (Selkon *et al*, 1979; Pflug and Odlaug, 1986).

For validated methods of sterilization with few variables, the efficacy of the process can adequately be measured by physical or chemical means. Measurement of temperature and time are adequate for

thermal sterilization processes, and radiation dose and duration for ionizing radiation sterilization. The use of biological indicators in these processes is therefore mainly restricted to validation procedures. In some of these cases, chemical indicators such as the black spot Browne's tube (Albert Browne Ltd, Leicester) or Thermolog-S integrators (PyMaH Corp., Somerville, New Jersey) are used. The Browne's tubes are known to be unreliable since they can change colour with a load, indicating sterility, that could then fail a sterility test. The integrator strips are supposed to be much more reliable, being based on a wax which migrates along a special paper. These latter types of indicator have been accepted in previous years by official bodies such as the Federal Drug Administration (FDA) as an indication of successful sterilization. However, more recently the FDA, in comparing several types of these indicators has given them an unfavourable report (The Microbiological Update, 1990).

In sterilization processes utilizing chemical sterilants in gaseous form, many more parameters are involved in the sterilization process. All of these must be monitored. In LTSF for example, not only the temperature of the chamber has an effect on the success of the cycle, but also the concentration, distribution, pressure and penetration of the formaldehyde. This problem also pertains to ethylene oxide (EtO) sterilization in which relative humidity also plays a part (Caputo and Rohn, 1988). Chemical indicators for monitoring such processes do exist, such as a paper indicator for the LTSF process (Albert Browne Ltd, Leicester), but more work is required to make them reliable (Line and Cutts, 1982). The use of biological indicators is therefore essential to integrate the effects of all the relevant parameters. BIs are used routinely to monitor the success of EtO sterilization (Caputo and Rohn, 1988). At

present, no suitable BI exists for use in LTSF sterilization, those in use were originally designed for monitoring wet heat sterilization, and are not reliable (Line and Pickerell, 1973). Until a reliable BI is available, LTSF will continue to be used as a disinfection rather than sterilization process by such organisations as the National Health Service (CSC report, 1986).

1.5 BIOLOGICAL INDICATORS

1.5.1 Definitions

a) Carrier

The material used to support the spores.

b) Test pieces

A carrier upon which a defined number of spores of a particular type has been deposited.

c) Primary pack

This is the container system of the test piece which has a defined permeability to the sterilizing agent or process to be monitored.

d) Biological indicator (BI) or Biological monitor (BM)

The test piece contained in its primary pack ready to monitor a sterilization process without further modification.

1.5.2 Properties of an Ideal Biological Indicator Organism

There are many properties an ideal indicator organism would possess, these are :

i) reproducible linear inactivation kinetics for the sterilizing process,

ii) non pathogenic,

iii) thermophilic, and easily identifiable,

iv) aerobic, easy to culture on a simple defined medium,

v) high Growth Index (i.e. high ratio of viable to non-viable spores),

vi) presentable to the sterilization process on a simple inert carrier system,

vii) more resistant to the sterilizing process than the most resistant likely contaminating organisms,

viii) quick and easy recovery to obtain fast results.

1.5.3 Development of Biological Indicators

The length of the list of properties it would be desirable for a BI organism to possess, means that it is unlikely that any one organism will possess them all to the desired amount. Unfortunately, it is also true that a good BI organism for one process is not necessarily any good for another process, for example the use of BIs designed for steam sterilization at above 115°C to monitor LTSF has proved unsuccessful (Line and Pickerell, 1973; Everall and Morris, 1977; Blake *et al*, 1977). For this reason, many different organisms have been tried and used for different processes. The first recorded use of a BI was by Koch in 1881 for the validation of a sterilization process. Since that time, research on the characterisation and development of various different organisms for different sterilization processes has been carried out. *B. stearothermophilus* spores are the most commonly used, generally for saturated steam sterilization at above 115°C (Kelsey, 1958; Kujala and Kauppinen, 1982). They have also been used for dry heat sterilization (Hobbs, 1980), and LTSF sterilization (Blake *et al*, 1977). Other organisms investigated include *B. pumilus* for monitoring U.V. sterilization (Abshire *et al*, 1983), *B. coagulans* for wet heat sterilization (Jones

and Pflug, 1981) and *B. subtilis* for EtO sterilization (Dadd and Daley, 1980; Myers *et al*, 1981; Dadd *et al*, 1983a; Gorman *et al*, 1983). In all the cases above the indicator organisms have been spores of the family *Bacillacae*. Others have been suggested, such as atoxigenic *Clostridium tetani* spores for heat sterilization (Dormandy *et al*, 1957) and use of *Cl. botulinum* in the food industry has been reported (Soper, 1988). In all these cases however, the organisms used have been spore formers, and it was the spores that were used. Spores are used because of their high resistance and for this reason they will be used in the development of a BI for use in LTSF. In a previous study by Hoxey in 1984, various *Bacillus* spp., including *B. coagulans*, *B. subtilis* and *B. stearothermophilus* were tried. None of these organisms appeared to fulfill enough of the criteria for a practical BI organism for LTSF. The greatest promise was shown by the *B. stearothermophilus* strains used as the other species were readily killed at temperatures employed in LTSF sterilization. For this reason, various strains of *B. stearothermophilus* sporulated on a range of defined media will be used in the present investigation.

1.6 CHARACTERISTICS OF BACTERIAL SPORE INACTIVATION.

In section 1.2.1, it was stated that it was thought that the manner in which micro-organisms exposed to lethal processes are inactivated could be described to a first approximation by first-order kinetics. However, this does not always appear to be the case and in inactivation experiments using bacterial spores, both linear and non-linear survivor curves for many different processes have been obtained. The types of survivor curve which have been obtained can be grouped into six categories (A-F), and these are illustrated in Figure 1.2.

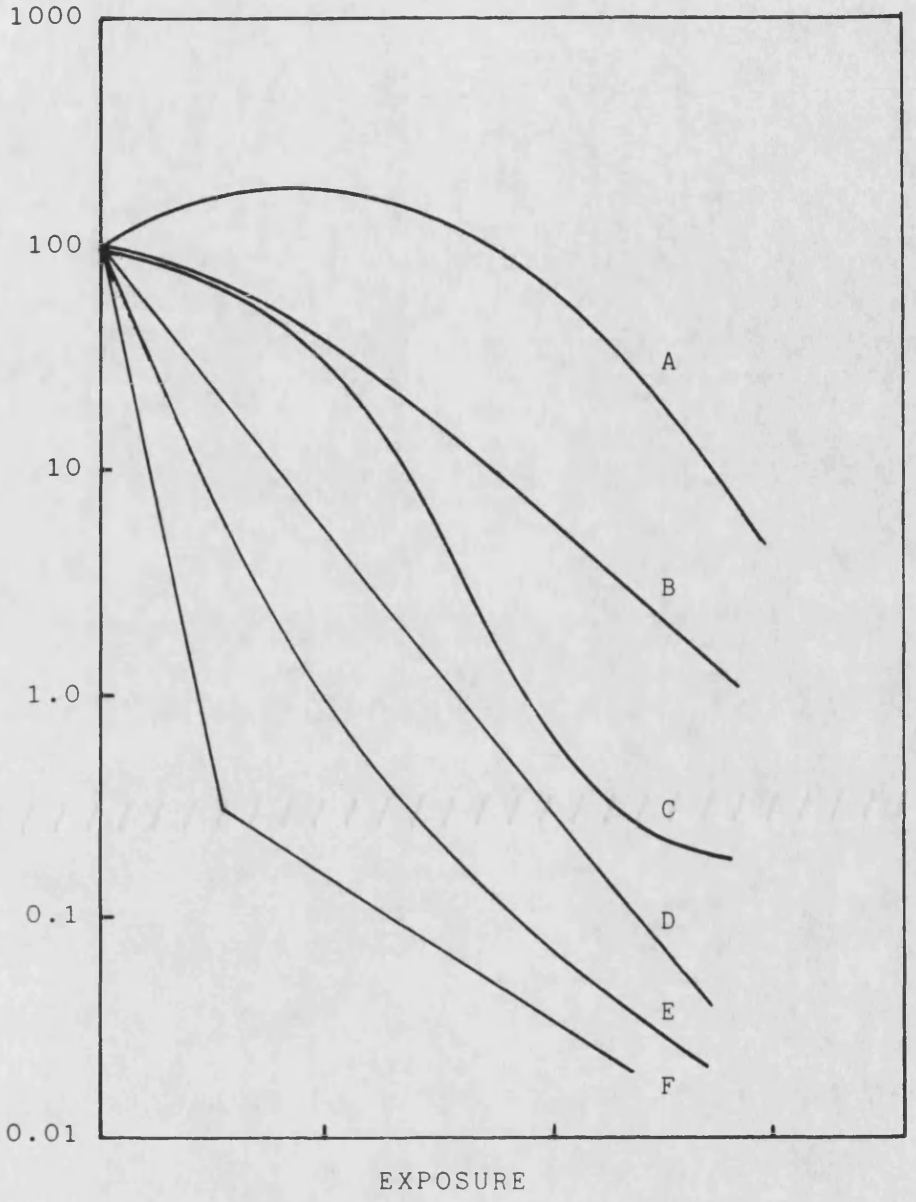


Figure 1.2 Types of Survivor Curves for the Inactivation of Bacterial Spores by Lethal Treatments.

Type A curves exhibit an initial activation above the t_o viable count. This is thought to be due to superdormant spores in the spore suspension being activated by the heat. This leads to an initial increase in viable count, prior to a decrease brought about by inactivation by the lethal agent. This type of curve is therefore likely to occur in heat inactivation of spore suspensions with low Growth Indices. Examples of heat activation which could lead to this have been reported for both *Bacillus cereus* (Keynan *et al*, 1965) and for *B. stearothermophilus* (Brown *et al*, 1968; Etoa and Michiels, 1988).

Type B curves exhibit an initial lag period, forming a shoulder, after which the inactivation proceeds in a log-linear fashion. Examples of this type of curve have been reported for dry heat inactivation of resistant spores (Hobbs, 1980). These shapes of curve are sometimes attributed to a combination of activation and inactivation (Cerf, 1977); or clumping (Stumbo, 1975). A mathematical relationship for this type of curve has been proposed (Alderton and Snell, 1970).

$$(\log N_o - \log N_t)^a = kt + c$$

where N_o = Number of organisms at time zero

N_t = Number of organisms at time t

t = Exposure time (time)

k = Inactivation rate constant ($time^{-1}$)

c = Constant

When "a" = 1 , the curve is log-linear. The value of k and the constant c can be derived respectively from the slope and the

intercept of the linearised survivor curve.

The sigmoidal curve depicted in Figure 1.2 Curve C, is considered to be caused by a combination of effects. The initial shoulder has the same explanation as for type B curve and the tail as for type E curves. It has been suggested that all survivor curves are of this type, and that absence of shoulder or tail is an artefact of the sample times chosen for the experiment.

Type D curves demonstrate the inactivation of a constant fraction of the bacterial population for unit time, and follow an exponential relationship. This is a first order kinetic inactivation and can be described mathematically as :

$$\frac{N_t}{N_o} = e^{-kt}$$

N_o = initial viable count; N_t = viable count at time t;

t = Exposure time; k = inactivation rate constant.

The inactivation rate constant, k, can be calculated from the slope of the survival curve as described in section 1.2.1. This is related to the resistance of the spore to the inactivating agent/conditions, with sensitivity increasing with higher k values.

Type E curves exhibit a decreasing inactivation rate with time. This could be due to one of two reasons, firstly that the inactivating process is becoming less lethal (e.g. concentration of sporocidal agent is dropping) or that there is a proportion of the population more resistant to inactivation. The former is almost certainly the case in LTSF inactivation experiments carried out by Chinyanganya (1989). The latter has not really been proven, and is discussed by comparison of the vitalistic and mechanistic theories of

resistance by Cerf (1977).

Type F curves are a special case, and are called biphasic curves. These more usually occur when the population being inactivated consists of two distinct resistances. This leads to a curve exhibiting two distinct D-values. An example of this is the resistance of *B. stearothermophilus* R and S variants which have been demonstrated to have different heat resistances (De Guzman *et al*, 1972). This would allow the construction of a biphasic survival curve such as the one illustrated by Cerf (1977).

The intercept ratio (IR) was proposed as a method to distinguish between different types of curve (Pflug and Holcomb, 1977). This is defined as :

$$IR = \log Y_o / \log N_o$$

Y_o = intercept on Y axis; N_o = initial viability of spores at t=0

When IR > 1 the curve is type A, B or C, if IR = 1 the curve is type D and if IR < 1 the curve is type E or F.

1.6.1 Resistance of the Bacterial Spore

1.6.1.1 Mechanisms of Heat Resistance

Though spore heat resistance has been recognised for a long time, the mechanism by which this is achieved is still not clearly understood. With the discovery of dipicolinic acid (DPA) by Powell in 1953, and the fact that it is only found in the spore protoplast (Leanz and Gilvarg, 1973), its role in heat resistance was hypothesized. Powell and Strange (1953) suggested that it was in fact a calcium-DPA complex that stabilized a water impermeable protoplast. The osmoregulatory theory of Gould, 1977 also attributed a significant role to DPA in spore resistance. However doubts were cast when in 1961, Walker *et al* showed that there was no direct

correlation between heat resistance of *Bacillus* spores and DPA content. They did, however, find some results to suggest that the ratio of DPA to Mg^{2+} and Ca^{2+} might have some effect though not in all cases. This dubious correlation was then even further discredited by Grecz and Tang, 1970 who showed there was no correlation between CaDPA and heat resistance. More recently it has been demonstrated that removal of CaDPA has no effect on spore resistance (Warth,1985). This taken with the evidence of Zytkovicz and Halvorson, 1972, who isolated DPA^- mutant spores which maintain their heat resistance seemed to prove that DPA had no role in heat resistance.

However, the role of DPA and in particular DPA chelates in heat resistance cannot be dismissed that easily. The results of Balassa *et al*, 1979 and Mallidis and Scholefield, 1985 have shown correlations between heat resistance and DPA chelates, and most recently Mallidis and Scholefield, 1987 have found evidence of DPA-Mn, DPA-Ca and DPA-Mg chelates being correlated with the heat resistance of five strains of *B. stearothermophilus*.

There are three main factors that are now accepted to affect spore resistance these are dehydration, thermal adaptation and mineralization.

i) Dehydration.

Many theories to explain spore heat resistance are based on the low water content of spores. It is well known that many proteins can be stabilized to heat by drying (Warth, 1985). Evidence that spore water content plays an important role in spore heat resistance is abundant. It has been demonstrated that the water content of *B. subtilis* var. *niger* was correlated with heat resistance, and that spores with water activity (aw) values of between 0.2-0.4 exhibited

maximum heat resistance , with decreases at values above or below this range (Angelotti *et al*, 1968). This was in agreement with Murrell and Scott (1966), who demonstrated greatest resistance at aw values between 0.2-0.4 for *B. coagulans, B. stearothermophilus, B. megaterium, Cl. botulinum* types E,B and C and *Cl. bifermentans*. It has also demonstrated that heat resistance of *B. stearothermophilus* is at maximum at aw 0.2, and that spores at aw 0.00 exhibited lower heat resistance than spores at higher aw values (Marshall *et al*, 1963; Murrell and Scott, 1966). It has been demonstrated that five species of *Bacillus* which exhibited a 600-fold difference in moist heat resistance owed their resistance in whole or in part to the extent of dehydration and diminution of the spore protoplast (Beaman *et al*, 1982). Perhaps more importantly this relationship held both within and between the species. Other workers have also demonstrated that spores with larger cortex and smaller protoplast are most resistance (Bayliss *et al*, 1981). More recently, Beaman *et al*, (1989) demonstrated that the low heat resistance of *B. sphaericus* was related to the high water content of its protoplast (approx. 61%) as measured by bouyant wet density techniques.

Assuming such an important role of protoplast dehydration in heat resistance, several hypotheses for the maintenance of spore dehydration have been proposed. Low water content in a system can be maintained by four possible mechanisms,

i) a water impermeable barrier,

ii) the constituents bind little water,

iii) the system is in equilibration with water vapour at RH < 100% or a solution of aw < 1.00,

iv) pressure exists between the system and one at high aw.

Each of these mechanisms are used by other life forms (Warth,

1985).

The first hypothesis can easily be disproven by demonstration of the permeability of the spore to water (Lewis *et al*, 1960) and other small molecules (Gerhardt and Black, 1961; Black and Gerhardt, 1962). The second hypothesis can be dismissed by the demonstration of the ready solubility of the contents (Warth, 1985). Finally mechanism three can be ruled out as spores are formed in aqueous media of high aw. This means that the only feasible mechanism of maintenance of spore dehydration is the exertion of pressure. Several possible ways by which spores could exert this pressure have been proposed including simple osmosis (Gould and Dring, 1975). More recently however, a more physical method, involving the cortex and perhaps the spore coats in "squeezing" the protoplast has been proposed. There are two models of this type, the Expanded Cortex Model and the Anisotropic Cortex Model. The Expanded Cortex Model relies upon expansion of the cortex uniformly as illustrated in Figure 1.3 (a), against both the protoplast and the spore coat. As the spore coat is considered to be mechanically strong, this expansion would be mostly directed at diminution of the protoplast. This would force water out of the protoplast leaving it dry. The anisotropic model, Figure 1.3 (b) rules out any role of the spore coat, and relies upon radial expansion of the cortex in layers parallel to the surface. This would exert pressure on the protoplast without the spore coat playing a restricting role. The latter of these two explanations is now accepted as more likely due to the lack of any major effect on the heat resistance of spores by removal of the spore coat, and the existence of resistant coatless mutants (Warth, 1978) supporting the anisotropic model. Further support for the anisotropic model is given by the results of Marquis *et al*, (1985) which demonstrated that

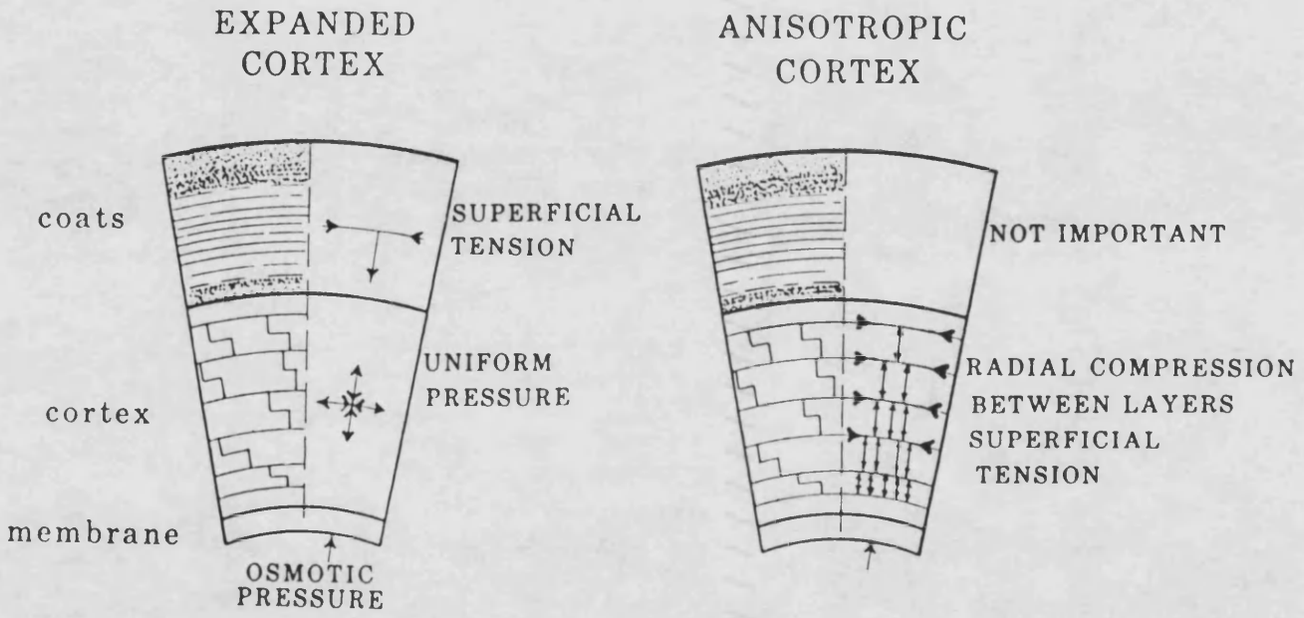


Figure 1.3 Diagrammatic Representation of the Forces Maintaining Dehydration of the Spore Cortex in the Expanded and Anisotropic Cortex Theories. (from Warth, 1985)

coats of spores of *B. megaterium* do not play a major role in protoplast dehydration, as their removal does not increase hydration. It appears that the spore coat only plays an auxillary mechanical restraining role, with the elasticity of the peptidoglycan in the cortex doing the majority of the work. Heat resistance has also been correlated to the development of a mature cortex and rudimentary coat in spore mutation studies (Gorman *et al*, 1984).

ii) Thermal adaption

Thermal adaption of organisms has been demonstrated by Warth, (1978) showing that there is a relationship between optimum and maximum growth temperature and heat resistance. This could be due to the production of extremely thermotolerant enzymes and structures. This can be achieved by adaptations at the protein level by simple amino acid substitutions and additional salt bridging (Yutani *et al*, 1977). Such enzymes have been isolated, for example an extremely thermotolerant neutral protease from *B.stearothermophilus* KP 1236 (Takii *et al*, 1987). This is probably a natural selection process, and the genes responsible have been isolated (Kubo and Imanaka, 1988). It has been demonstrated that another method of thermal adaptation is to change the intracellular ion concentration (Hensel and Konig, 1988).

iii) Mineralization

Spores are highly mineralized with Ca^{2+}, but also with other minerals such as Mn^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} though they generally contain less K^{+} than vegetative cells. Beaman and Gerhardt (1986) studied the resistance of 28 types of spores amongst seven species of *Bacillus*. These organisms came from a range of thermophiles,

mesophiles and psychrophiles exhibiting a 3000 fold range in heat resistance. This study revealed that there was a relationship between mineralization, thermal adaptation and dehydration at between 28% and 57% water content (wet weight basis). Below this, increases in mineralization and thermal adaptation increased heat resistance independently of protoplast water content. This is probably the best single demonstration of the importance of all these factors in heat resistance.

Although there seems little doubt that mineralization, dehydration and thermal adaptation play major roles in the production of spore resistance, there are other mechanisms still to be investigated. One of these is the presence of three acid soluble proteins (SASP) denoted α, β and γ. Mason and Setlow (1986) have demonstrated an essential role of α and β SASP in U.V. resistance in *B. subtilis* spores. But it also appears that the α and β SASP have interchangeable roles in U.V. and heat resistance of *B. subtilis* (Mason and Setlow, 1987). The SASP does not appear to play a detectable role in the resistance of the spores as demonstrated by SASP γ^- and γ^+ mutants (Hackett and Setlow, 1988).

1.6.1.2 Mechanisms of Chemical and Radiation Resistance.

It has been suggested that the low water activity of spores, which partially accounts for heat resistance may also be responsible for chemical resistance (Waites, 1983). For example it has been demonstrated that resistance of spores of *B. subtilis* var *niger* to ethylene oxide increases with decreasing water content (Dadd *et al*, 1985). However, this does not necessarily mean that spore resistance to one chemical can be related to heat or U.V. resistance (Bayliss *et al*, 1981).

Several studies on the development of chemical resistance have been carried out. These have concentrated on relating morphological change during sporulation to development of resistance. The designation of the various stages of spore formation are shown in Figure 1.4 This, as stated earlier is difficult, as the designation of spore stages are arbitary as sporulation is a continuous process. Also, not all spores will be synchronized in sporulation. It is possible to block sporulation at one of the stages by insertion into the regulatory gene (Errington *et al*, 1988). However, in most of these studies, spore mutants blocked at one of these stages were used. From these studies, it has been found that there is developmental sequence to aquisition of resistance to various chemicals, heat and U.V. Gorman *et al*, (1984) demonstrated that heat resistance emerged at stage V with chemical resistance (glutaraldehyde, hypochlorite and iodophore), developing in middle to late stage V depending on the chemical. The sequence was heat, povidone iodine, glutaraldehyde, hypochlorite. The development of resistance to glutaraldehyde, hypomethanol, chlorine and povidone iodine appeared to be related to emergence of phase brightness, uptake of ^{45}Ca and DPA and cortex formation, giving credence to heat resistance mechanisms helping in chemical resistance. Other workers have demonstrated sequential development of resistance to chemicals. Power *et al*, (1988) showed that toluene resistance evolved at stage III - IV, heat at early V, lysozyme at middle V and glutaraldehyde at late V in *B. subtilis* 168 spores. Chlorhexidine resistance has been shown to develop after toluene resistance but before heat resistance (Shaker *et al*, 1988). It has also been demonstrated that resistance in *B. subtilis* occurred sequentially in the order octanol, butanol, chloroform-methanol,

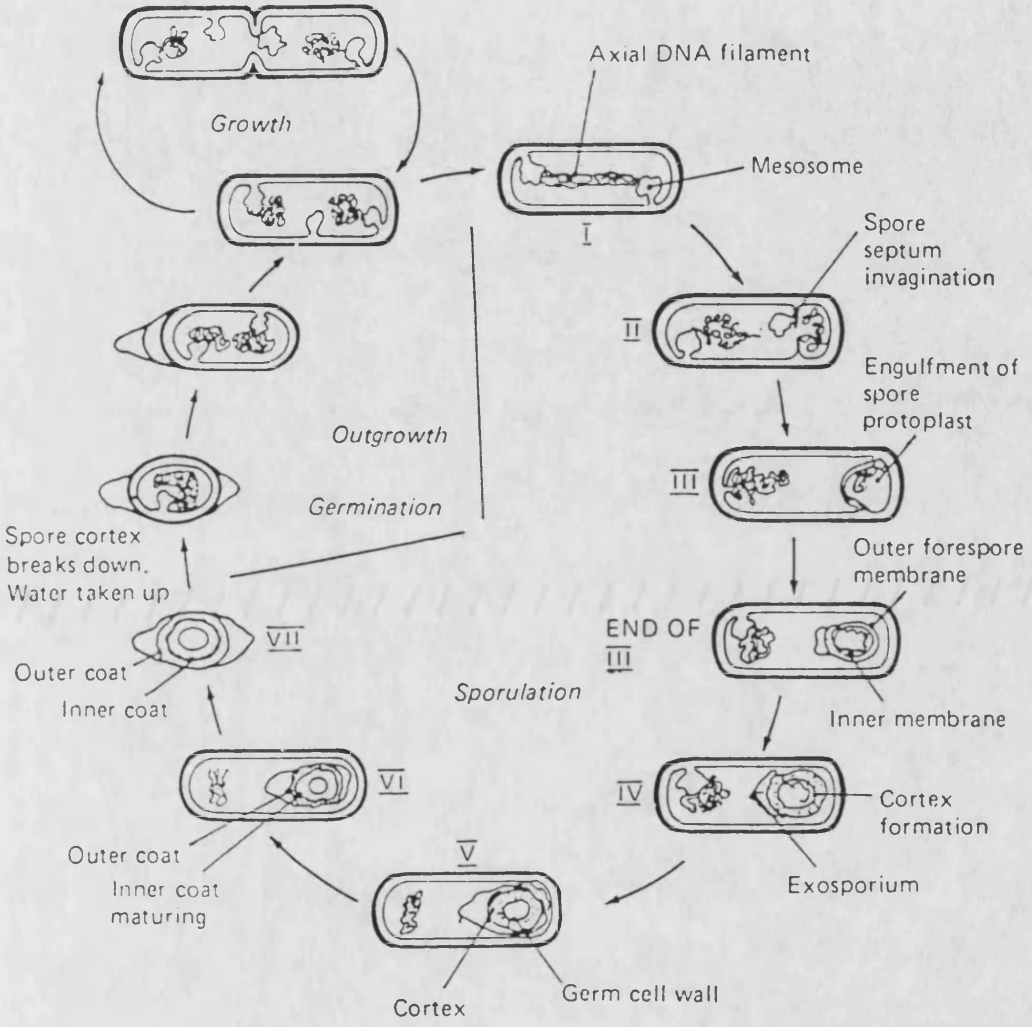


Figure 1.4 Morphological Stages During Sporulation and Germination in Bacillus Species from Dawes and Sutherland, 1980.

ethanol and phenol (Balassa *et al*, 1979), but all of these resistances occurred before onset of heat resistance. This is in agreement with the findings of Sousa *et al* that resistance to xylene, toluene, octanol and chloroform all developed before resistance to heat (Sousa *et al*, 1978).

The role of the spore coat in protection of spores against chemicals is not in doubt. Evidence for its role in resistance to ethylene oxide (Dadd and Daley, 1982), hypochlorous acid (Gorman *et al*, 1984), and octanol (Balassa *et al*, 1979), have been demonstrated. The role of the coat in resistance to Betadine at 37°C was also demonstrated by increasing susceptibility correlating with increasing coat damage (Gorman *et al*, 1985). Removal of the coat and cortex has also been demonstrated to affect spore resistance to chlorine and proves their role in resistance, though it may be mediated through their role in maintenance of protoplast dehydration (Bloomfield and Arthur, 1989).

Although as mentioned earlier the spore coat is permeable to a great extent to small molecules (Gerhardt and Black, 1961), it nevertheless stops the penetration of some molecules due to the impermeability of its disulphide rich protein and alkali soluble protein (Russell, 1982). This is demonstrated by the evidence that breaking of these bonds increases the susceptibility of *Cl. bifermentans*, *B. subtilis* and *B. cereus* spores to hypochlorous acid (Wyatt and Waites, 1975), and also increased spore sensitivity to hydrogen peroxide (Gould and King, 1969). Even molecules which can easily penetrate the molecular size of the spore coat and cortex can be impeded by these structures. This can occur by providing sites of, for example, alkylation which will react with a compound such as ethylene oxide and hence acts like a sponge stopping these molecules

penetrating further to more vital components in the protoplast. The idea was suggested to account for ethylene oxide resistance being correlated to the existence of a spore coat (Dadd and Daley, 1982).

1.6.2 Factors Influencing Bacterial Spore Resistance

For the production of spores for use in Biological Indicators, conditions should be defined so as to produce spores of reproducible characteristics. For this reason, the many factors that can influence spore resistance should be considered.

These factors can be classified into four main groups (Kujala and Kaupinen, 1982).

i) Genetic factors

ii) Factors during growth and sporulation.

iii) Factors during spore harvesting and storage.

iv) External factors during inactivation and recovery.

1.6.2.1 Genetic Factors

It must be understood that the classification of species of bacteria is artificially designed, based on physiological and biochemical studies. This means that although bacteria within a particular species will possess common characteristics, as defined in Bergey's manual of determinative bacteriology (1975), there is a great deal of scope for variation (Molin, 1982). Roberts (1968) demonstrated that the D_{90} values of *Cl. welchii* could vary by up to 48 fold. More recently, Priest *et al*, (1988) demonstrated the heterogenicity of six species of *Bacillus* including *B. brevis, B. sphaericus and B. stearothermophilus* on the basis of phenotype and molecular genetic data. *B. stearothermophilus* is considered to

encompass at least three distinct taxa, and the genus *Bacillus* to encompass some 80 taxa in all (Priest *et al*, 1988). The heterogenicity of *B. sphaericus* as determined by genetic and phenotypic data has also been demonstrated (Carboulec and Priest, 1989). Many authors have demonstrated that different strains of the same species can exhibit different characteristics when grown under identical conditions (Black *et al*, 1977; Hoxey, 1984).

Despite this heterogenicity, generally, thermophilic strains are more resistant than mesophilic or psychrophilic ones, particularly in response to lethal heat treatments (Beaman and Gerhardt, 1986).

1.6.2.2 Factors during Growth and Sporulation

Influences during growth and sporulation that can have an effect on spore characteristics can be divided into two main categories. Firstly there is the composition of the growth/sporulation medium, and secondly the conditions under which the organisms are incubated.

a) Nutrients

There has been a great deal of effort invested in the production of bacteria and spores with stable, reproducible characteristics. Defined media have been described for use in the study of vegetative bacterial physiology including media for *B. subtilis* (Demain, 1958; Fang and Demain, 1989) and *Clostridium* spp. (Lovitt *et al*, 1987a, b; Sedden and Borriello, 1989). It has been demonstrated that the use of chemically defined media is the most suitable method of production of *B. subtilis* spores with minimum batch to batch variation in characteristics (Hodges *et al*, 1980), though this does not guarantee that these characteristics will be stable in storage (Alpin and Hodges, 1979). Several media have been described for the production of spores with reproducible characteristics (Anderson

and Friesen, 1972; De Guzman *et al*, 1972; Hodges *et al*, 1980), and have been used to produce organisms for screening as potential Biological Indicators as discussed in 3.1. There is a lot of conflicting evidence for the role of specific nutrients in the sporulation of bacteria and on the yield and characteristics of the final spores. It has been reported that spore yield for *B. stearothermophilus* can be increased by adding glucose to sporulation media up to a maximum concentration of 17 g/l (Kujala and Kauppinen, 1982). It has also been reported that low concentrations of glucose with high concentrations of L-glutamic acid in the medium will increase spore yield (Anderson and Friesen, 1972). Calcium is thought to help increase spore resistance by stabilization of enzymes in conjunction with DPA (Mallidis and Scholefield, 1987). The inclusion of manganese ions in the sporulation medium has also been reported to increase the resistance of various *Bacillus* species (Tallentire and Chiori, 1963; Aoki and Slepecky, 1973). This could also be due to the formation of DPA complexes (Mallidis and Scholefield, 1987). Whatever the evidence for individual effects of the medium components it has been demonstrated that media can effect the production of spore macromolecules such as tRNA (Lazzarini and Santangelo, 1967), and components of spore structure such as lipid and protein. It can also have an effect on spore coat properties (Bayliss *et al*, 1981; Cheung and Brown, 1985) and these are known to have an effect on spore resistance to chemicals such as hypochlorite (Waites and Bayliss, 1979). Changes in spore coat properties could lead to increased lag time on survival curves (Yokoya and York, 1965) or changes in spore hydrophobicity (Foegeding and Fulp, 1988).

b) Incubation Conditions

Warth (1978) demonstrated that there was a relationship between incubation temperature during sporulation and the heat resistance of the spores produced, with increasing temperature increasing the heat resistance of thermophilic organisms. For mesophilic, aerobic spore formers however, the lowest temperature that will produce good growth gives spores with maximum heat resistance (Hurrell, 1988).

Alteration of sporulation temperature is known to effect the lipid content of the spore, and it has been suggested that DPA/cation ratio may be affected (Russell, 1987). It is also known that different sporulation temperatures have an effect on the structure of *B. stearothermophilus* spores, with low growth temperature (45°C) favouring the rough variant and high temperatures (55°C) favouring the smooth variant (Hill and Fields, 1967). There may be structural differences between these varients which could have an effect on their resistance characteristics.

However, not all evidence demonstrates a link between sporulation temperature and spore resistance. Spores of several *Bacillus* species have been shown not to be affected in their resistance to ethylene oxide (Dadd *et al*, 1983a) and heat (Rey *et al*, 1975) by the sporulation temperature.

c) pH Effect

pH has been demonstrated to have an effect on the sporulation of *B. stearothermophilus*, with strict pH control at pH 6.8 reported to give rise to maximum production of spores (Kujala and Kauppinen, 1982). Despite this there appears to be no correlation between the pH of the sporulation medium and the heat resistance of the spores (El-Bisi and Ordal, 1956).

d) Effects of Spore Harvesting

Many methods have been proposed for producing good yields of free cleaned spores. However, many of these methods are known to have an effect on the resistance of the spores. To release spores from the sporangia, lysozyme has been used by several workers. This has been demonstrated to alter spore resistance to chemical insult, possibly by altering spore coat permeability (Gould, 1985). Other methods of releasing spores include induced release by sodium sulphate (Burnett *et al*, 1986), or ultrasonication, (Goodenough and Solberg, 1972). In the case of ultrasonication, the technique has been shown to have detrimental effects on spore resistance (Ordonez and Burgos, 1976; Sanz *et al*, 1985; Garcia *et al*, 1989). No work on the effect of sodium sulphate on spore resistance has been reported.

After spores have been released they must then be cleaned to remove media and vegetative debris. The simplest method for this is multiple washing by centrifugation (Long and Williams, 1958). However even this simple technique, if excessive, can affect spore resistance (Gorman *et al*, 1984). Other methods of cleaning have included density gradient centrifugation (Prentice *et al*, 1972) and two phase separation (Sacks, 1969). Both of these methods use chemicals, whose effects on spore resistance are not known. Heat shocking spore suspensions to kill any remaining vegetatives has been shown to have an effect on resistance to ethylene oxide (Dadd *et al*, 1983a), possibly by altering spore coat permeability to ethylene oxide or by altering the tertiary structure of the protein responsible for dormancy.

e) Storage

For spore preparations to be considered as potential biological indicators for sterilization processes, they must have stable

characteristics during storage. The evidence for the effects of storage on bacterial spore resistance is sometimes contradictory. Loss of moist heat resistance of *B. stearothermophilus* spores has been reported with storage at room temperature (Cook and Brown, 1965a; Reich *et al*, 1979). Cook and Gilbert (1968) reported exactly the opposite however. Alpin and Hodges (1979) demonstrated a marked decrease in heat resistance and viability of *B. stearothermophilus* spores after freezing and freeze drying, and an increase in resistance when stored in aqueous suspension at 22°C and 4°C for three months. Reich (1980) reported no effect on the resistance of *B. subtilis* spores to ethylene oxide after storage at room temperature for up to 24 months. More recently Leaper and Bloor (1988) reported that storage of spores of *B. subtilis* or *B. subtilis globigii* for up to 144 weeks at 4°C had no effect on their resistance to hydrogen peroxide, though resistance to peracetic acid stayed the same for the former and increased with time for the latter. From these results it can be seen that various workers store spores under different conditions making comparisons very difficult. However in general it has been suggested that spore water activity, which is known to have an effect on spore heat resistance (1.6.1.1), is related to spore age (Beloian, 1977).

1.6.2.3 External Factors During Inactivation and Recovery

a) Presentation to Inactivating Agent

Spores are generally presented to sterilizing conditions either dried onto a carrier material, or suspended in a liquid medium. Both of these methods have been demonstrated to affect spore resistance. In the case of a solid carrier system, the material of which it is composed has been shown to affect the dry heat resistance of spores

(Angelotti *et al*, 1968), possibly by altering how fast the spore dehydrates. It has been demonstrated that the method of drying the spores and their final water activity can affect their resistance to lethal agents (Myers *et al*, 1981; Dadd *et al*, 1983b).

For liquid suspensions, both the pH and the constituents of the medium could have an effect on spore resistance. The constituents might either aid or interfere with the lethality of the sterilizing agent (Cook and Gilbert, 1965; Russell and Harris, 1968). Sodium chloride at concentrations of 2, 4 and 8% w/v in the suspending medium has been shown to reduce the heat resistance of *B. stearothermophilus* spores but had no effect on heat or radiation resistance of spores of *B. pumilus, B. subtilis* or *B. subtilis* var. *niger* (Briggs and Yazdany, 1970). Buffers used to control the pH of the suspending medium can also have an effect on the lethality of a sterilant (Pflug and Smith, 1977). There are conflicting reports of the effect of pH. It appears that pH's in the range 5-9 have least effect on resistance (Roberts and Hitchins, 1969). However pH 7.2 has been reported to increase *B. stearothermophilus* spore resistance from that obtained at pH 6.5 (Harnulv *et al*, 1977). Gould (1985) suggested that protonation of the spore components, such as the spore peptidoglycan, may result from low pH, and this could have an affect on its role in maintaining spore dehydration. It has also been reported that acidic conditions can cause release of DPA and activate spores, resulting in reduction in resistance (Brown *et al*, 1968).

In conclusion, the method of presentation and preparation of spores for inactivation studies is very important. Cleanliness (Doyle and Ernst, 1968), dessication and pH must be taken into account, along with any interactions between the supporting medium

constituents and the spore or inactivating agent.

The shape of the carrier or test piece can also have a significant effect, for example with formaldehyde it can alter the degree of penetration of the sterilant gas (Spicher and Borchers, 1983).

b) Recovery of Injured Spores.

The measurement of the resistance of a bacterial population to insult, be it vegetative cells or spores, relies upon the ability of viable cells/spores to replicate to form visible colonies (Russell, 1982). It is therefore very important to recover the compromised organism under the most favourable conditions to prevent overestimation of the bactericidal ability of a process. In general, damaged spores require lower concentrations of chemical inhibitors to prevent growth (Roberts, 1970). A spore, whether injured or not must germinate through a development sequence which is unique to spore formers. This includes genetic, metabolic and morphological changes. Injury may be expressed or repaired at any of these stages (Gould, 1984). This development, being complex, is very easily damaged. The greater the damage, the more sensitive the spore. This is demonstrated by the increased inhibition of *B. stearothermophilus* spore recovery by the presence of NaCl in recovery medium (Briggs and Yazdany, 1970). Other media constituents such as long chain fatty acids and thioglycollate are inhibitory to growth and germination of some *Clostridia* (Roth and Halvorson, 1952; Segner *et al*, 1966; Ernst, 1968).

The pH of the recovery medium is also important, with the optimum pH for recovery of *B. stearothermophilus* spores reported as pH 7.4 (Cook and Brown, 1965b; Roberts, 1970) and pH 6.9 for *B. subtilis* (Dadd *et al*, 1983).

The temperature of incubation can affect recovery. It has been reported that the optimum temperature of recovery of heated *B. stearothermophilus* spores is 45-50°C, which is below the optimum of 56-60°C reported for untreated spores (Cook and Gilbert, 1968). The optimum recovery temperature for spores of *B. subtilis* has been reported to be 30°C, which is also lower than the optimum for untreated spores (Prentice and Clegg, 1974).

A major factor in recovery of spores after inactivation by chemical agents can be the pretreatment of the spores prior to plating on the recovery medium. Spicher and Peters (1976; 1981) demonstrated enhanced recovery of various *Bacilli* after treatment prior to plating. This effect has also been obtained by Gorman *et al* (1984) when inactivating *B. subtilis* with hypochlorite, though not to such a great extent. Other pretreatments have been reported to increase the recovery of spores after chemical inactivation, including treatment with alkali (Dancer *et al*, 1989; Power *et al*, 1989), lysozyme (Gorman *et al*, 1983; Power *et al*, 1989), abrasion and ultrasonication (Gorman *et al*, 1983), proteases and sodium hydroxide (Power *et al*, 1990) There have been varying reports of success with these treatments and with combinations of them, though none obtained recoveries of the magnitude of those reported by Spicher and Peters (1981) using sub-lethal heat.

1.7 PHYSICO-CHEMICAL PROPERTIES OF FORMALDEHYDE

Synonyms : Methanal, Formic aldehyde, Methyl aldehyde

Formaldehyde is a colourless, flammable gas and is characterised by a pungent odour and causes irritation of the mucous membranes at concentrations as low as 0.24 ppm (Pabst, 1987). The gas can cause

inflammation of the eyelids and irritation of the eyes (Wartew, 1983). Both gaseous and liquid formaldehyde will polymerise rapidly at room temperature and below to form a white solid precipitate of polyoxymethylenes of varying chain length, containing 90-99% formaldehyde and a portion of combined and free water. Polymeric forms of formaldehyde will gradually vapourise, at a rate dependant on temperature, to form monomeric formaldehyde gas.

1.7.1 Formaldehyde in Solution

Formaldehyde is only sparingly soluble in non-polar solvents such as toluene or chloroform. It is readily soluble in all polar solvents with the reaction being exothermic. The energy generated by solution in water is 3.55 KJ g^{-1} $mole^{-1}$ (Walker, 1953).

Dissolved formaldehyde is present principally as the monohydrate, methylene glycol ($CH_2(OH)_2$) along with a series of low molecular weight polymeric hydrates. Increasing the formaldehyde concentration in solution shifts the equilibrium towards the higher molecular weight polymers. Increase in temperature has the reverse effect. Due to the ready precipitation of solid polymers out of solution, commercial formaldehyde solutions (Formalin) contain 37-40% HCHO in aqueous solution with 10-15% methanol present as a stabilizer to prevent precipitation.

1.7.2 Health Hazards of Formaldehyde

The dangers to health of formaldehyde inhalation by laboratory workers was emphasised many years ago. It is a very widely used chemical, and is contained in adhesives, shampoos, carpeting, car exhausts and tobacco smoke (Wartew, 1983).

1.7.2.1 Toxicity of Formaldehyde

Occupational exposure is likely to occur through contact with skin, eyes, ingestion and most commonly inhalation (Wartew, 1983). The eyes as mentioned in 1.7 are very sensitive to low concentrations of the vapour. At levels of 25-50 ppm actual tissue damage can occur. Allergic reactions such as dermatitis are common with regular skin contact (Pabst,1987), and severe exposure can cause tanning of the skin. Ingestion is relatively uncommon, and the lowest published LD_{50} for humans is 36 mg/kg. Formaldehyde has been reported to cause pulmonary oedema, pnuemonitis and death by inhalation (Wartew,1983). However it is rapidly metabolized in the body, as it is normally ingested in such food as fruit, and its half-life is 1.5 minutes (Pabst, 1987).

1.7.2.2 Mutagenicity and Carcinogenicity of Formaldehyde

Formaldehyde is recognised as a mutagen for bacteria (Auerbach *et al*, 1977), but few studies have been carried out on mammals. Formaldehyde is known to cause squamous cell carcinomas of the rat nasal cavity (Swenberg *et al*, 1980), which may be caused by inducement of DNA-protein cross-links (Cosma *et al*, 1988). However these tumours were induced by exposing rats for a large proportion of their lifespan to levels of formaldehyde that no human would tolerate. Formaldehyde will induce chromosomal aberrations in mammalian cells when grown *in vitro* (Natarajan *et al*, 1983). It has been shown that there is no increase in mortality due to cancers of any kind in populations which are exposed to formaldehyde (Purchase, 1985), and for this reason many think that the risk associated with formaldehyde exposure is overstated (Pabst, 1987).

1.7.3 Occupational Exposure Limits of Formaldehyde

The long term maximum exposure limit in the U.K. (8h Time Weight Average Reference Period) is 2 ppm and the short term (10 min) maximum is also 2 ppm (COSHH, 1990). This is more stringent than the previous 10 ppm limit. This means that the average exposure limits would have to be below 2 ppm for most of the day to meet these requirements (Purchase, 1985). Other countries have set different limits for example the U.S.A. have a 5 ppm limit with a maximum of 10 ppm for up to 30 min in every 8 h (Blackwell *et al*, 1981).

1.7.4 Measurement of Formaldehyde

The detection and measurement of a compound relies upon the reactivity and other properties of that compound. The chemical reactivity of formaldehyde, such as its pronounced reducing action in alkaline solution, and its characteristic derivatives, provide a wide choice of assay methodology. For the assay to be useful, it must be specific, convenient, fast and simple to use, and of high sensitivity. Many of the assays for formaldehyde do not meet one or more of these requirements and may even react to other aldehydes and unrelated compounds. An example of this is that sulphur dioxide will interfere with the p-phenylenediamine assay for formaldehyde. Various methods have been used in practice, such as the chromotropic acid method (Altshuller *et al*, 1961; Hurrell *et al*, 1983; Gibson *et al*, 1968), the sodium sulphite method (Chinyanganya, 1989), high performance liquid chromotography (Mansfield *et al*,1977), and gas chromatography (Dumas, 1982).

In LTSF sterilization the sterilant gas contains a mixture of formaldehyde, steam, methanol, and a small amount of air. The most

convenient method of analysing this mixture would be gas chromatography (GC) as it would require no modification of the gas sample. Unfortunately, it has been demonstrated that of four types of GC detectors assessed, thermal conductivity and flame ionization detectors were not sufficiently sensitive (Committee on Aldehydes, 1981), photo-ionization detectors were sensitive but only with a high energy lamp which shortened the life of the detector (Dumas, 1982) and the electron capture detector only produced a limited linear response (Committee on Aldehydes, 1981). Gas-liquid chromatography (GLC) is also a convenient assay but it requires dissolving the formaldehyde out of the gas sample into a solvent. The drawback of GLC is that the small size of the formaldehyde molecule means that it is eluted from the column very rapidly. This will only give a very small response in a flame ionization detector, and even less in a thermal conductivity detector (Committee on Aldehydes, 1981). High performance liquid chromatography (HPLC) can be used to analyse formaldehyde once it is in solution but this requires derivatising the formaldehyde with 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone (Mansfield *et al*, 1977). The major drawback of this method is that it takes 24 hours to form the derivative and extract it into chloroform so that it may be assayed in a HPLC. Improvements in recent years have been able to reduce this time to 10 minutes, but it is still not a fast method (Benassi and Semenzato, 1989).

Some workers have attempted to develop spectrofluorometric methods for assaying formaldehyde. Belman (1963) reported very low detection limits of around 0.005 µg/ml for the acetylacetone fluorometric assay of formaldehyde. This is a ten-fold higher detection sensitivity as compared to the MBTH spectrophotometric assay. Unfortunately,

spectrofluorometric techniques are very sensitive to the design of the detection instrument, and give varying results as a consequence. An example of this is that Sawicki *et al* (1963) reported a detection limit of 1.2 ug/ml for the same acetylacetone fluorometric assay method as Belman. Also problems with non linear calibration lines have been reported (Belman, 1963).

Another method under consideration is based on the chemiluminescence reaction of formaldehyde with gallic acid in the presence of alkaline peroxide (Slawinska and Slawinski, 1975) though this requires proper design of the flow system and optical cell, which is expensive and involved.

Probably the most commonly used methods for accurate determinations of microgram quantities of formaldehyde are spectrophotometric (Table 1.1). Of these, chromotropic acid is one of the most widely used, and has been used to assay formaldehyde samples from inside a LTSF autoclave (Marcos and Wiseman, 1979; Hurrell *et al*, 1983). The major problem associated with spectrophotometric methods of assaying formaldehyde is the wide variety of substances that can interfere with the assay (Table 1.1). A comparison of the four most suitable methods in terms of sensitivity and specificity to formaldehyde was carried out by Hoxey (1984). The 3-methylbenzothiazalinone hydrazone (MBTH) assay was considered the most effective. The MBTH assay was developed by Sawicki *et al*, 1961 and modified for increased sensitivity by Hauser and Cummins (1964). The assay reagent (3-methylbenzothiazolinone hydrazone hydrochloride (MBTH)) is used as the entrapment agent for formaldehyde gas.

1.7.5 Inactivation of Formaldehyde

To investigate the effect of the exposure of bacteria to an

Table 1.1 Summary of Spectrophotometric Methods for Analysis of Formaldehyde in Ambient Air (Modified from Committee on Aldehydes, 1981).

Method	Minimum Detectable Concentration (μg/ml)	Absorbance Wavelenngth (nm)	Interferences
Chromotropic acid	0.25	580	Nitrogen dioxide Alkenes, acrolein acetaldehyde, phenol
Pararosaniline	0.1	570	SO_2, Cyanide
	0.1	560	Virtually specific
J-acid	0.3	468	Formaldehyde precursors
Phenyl-J-acid	0.4	660	Formaldehyde precursors
p-phenylenediamine	1.7	485	SO_2, aliphatic aldehydes
Tryptophan	0.15	575	Virtually specific
MBTH	0.05	628	Higher aliphatic aldehydes
Purpald	0.15	549	Higher aldehydes
Acetylacetone	1.4	412	Specific (?)

inactivating process, it is necessary to ensure the negation of the biocidal effect immediately at the sampling time. In the case of physical sterilizing agents (heat and radiation), the removal of the source, or in the case of heat, rapidly cooling the sample will achieve this. For chemical antimicrobial agents with a high concentration exponent e.g. phenol, diluting of the inactivating agent is sufficient. For formaldehyde however, with a concentration exponent of 1.0 (Russell, 1987), it is necessary to inactivate the formaldehyde. This is particularly necessary not only because of its sporicidal activity (which would be negligible at room temperature) but also because it inhibits spore germination and is bacteriostatic, preventing outgrowth. The use of inactivators therefore leads to a more reproducible quantitative assessment of the effect of the antimicrobial agent (Cheung and Brown, 1982).

Several methods to inactivate formaldehyde have been reported, including centrifugation and washing (Spicher and Peters, 1976) and filtration (Hayes *et al*, 1982). These are purely physical methods of removal. Chemical methods reported include morpholine and dimedone (Nash and Hirch, 1954), sulphite (Nordgren, 1939), and glycine (Nash and Hirch, 1954; Hoxey, 1984; Chinyanganya, 1989). In choosing an inactivator, it is important that neither the reaction product of inactivator and bactericide nor the inactivator itself is inhibitory to any of the processes of spore growth (including germination and outgrowth). Although glycine is not the optimal choice (Hurrell, 1988) because of its known inhibitory effect, it was found to be the preferred choice as it removed all active formaldehyde, and it can be successfully removed by filtration and washing (Hoxey, 1984; Chinyanganya, 1989). This is preferable to the inactivation of spores brought about by some other amino acid inactivators, and the

fact that they left free formaldehyde, itself sporicidal and inhibitory (Hurrell, 1988).

1.8 HISTORY OF FORMALDEHYDE AS A BACTERICIDE

Formaldehyde's bactericidal properties were first reported in 1886 by Loew and it was subsequently used as a fumigant for disinfection of large rooms (Aronson, 1897). Formaldehyde is unacceptable for sterilization as a gas alone, due to its poor penetrability and poor sporicidal activity (Christensen and Kristensen, 1982). It is still used as a fumigant at room temperature, and work is continuing to improve its effectiveness (Cross and Lach, 1990). The first use of formaldehyde at elevated temperatures was that of Sprague (1899), who demonstrated the inactivation of *B. anthracis* spores in a vacuum chamber using formaldehyde at 90°C. If however, formaldehyde is used in conjunction with sub-atmospheric pressure steam, it becomes a more effective and reliable process (Esmarch,1902; Nordgren, 1939). Nordgren reported that gaseous formaldehyde was more effective in an atmosphere of low relative humidity (50%). This is not always so for formaldehyde sterilization as it is the reverse of that reported for gaseous formaldehyde at room temperature (Cross and Lach, 1990) who reported greater activity at higher RH values (Figure 1.5).

The bactericidal activity of aqueous solutions of formaldehyde was demonstrated by Tilley (1945), who showed concentrations of 0.166-4% aqueous formaldehyde to be active against a range of organisms including *E. coli, E. bertheue* and *Staph. aureus*. Sporostatic and sporicidal activity of aqueous solutions of formaldehyde was demonstrated by Trujillo and David (1972) against spores of *B. subtilis* var. *niger*.

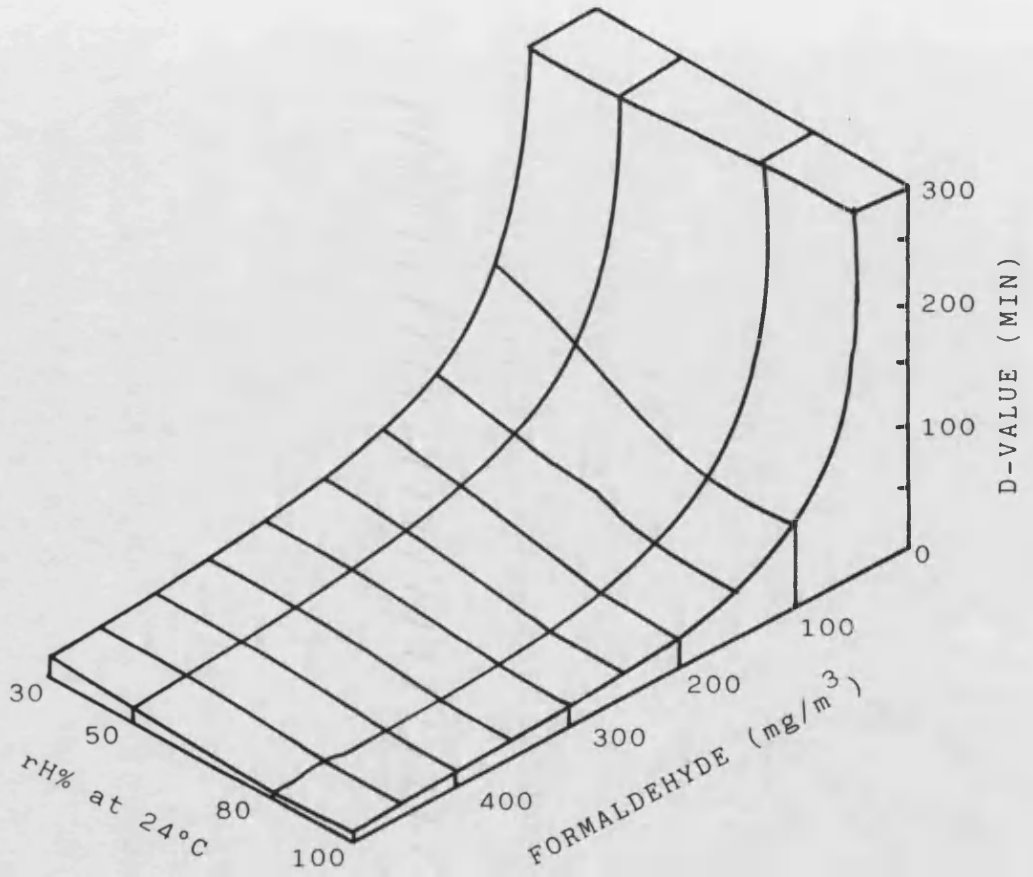


Figure 1.5 Affect of Relative Humidity and Formaldehyde Concentration on the D-value of Spores.

from Cross and Lach, 1990b

1.9 MECHANISM OF FORMALDEHYDE LETHALITY

1.9.1 General Action

As an alkylating agent, formaldehyde will react with a wide variety of macromolecules and precursors of macromolecules. It has been shown to react with primary amino groups in protein molecules (Fraenkel-Conrat, 1945) to give a more dense, less permeable structure (Fraenkel-Conrat and Mecham, 1949). It will also react with nucleotides, RNA and denatured DNA to give monomethylol derivatives, with protein and nucleic acids to give methylene cross-links, and with nucleoprotein to form protein nucleic acid cross-links (Benyajati *et al*, 1983; Bedford and Fox,1981; Wilkins and MaCleod, 1976). However, formaldehyde will not react with natural DNA, without the interstrand H^+ bonds being broken first (Chattoraj, 1970; Kozlov and Debabov, 1972). It has been shown that such breakage between the strands does occur naturally below the denaturation point of DNA (Luckashin *et al*, 1976). This frequency of occurence of such breakages increases with increasing temperature. Once the bonds are broken, formaldehyde will prevent bond reformation (Chattoraj, 1970). Formaldehyde has long been known to be a mutagenic compound and this has been demonstrated for *E. coli*, *Pseudomonas fluorescens* (Englesberg, 1952) and *Salmonella typhimurium* (Temcharoen and Thilly, 1983). Formaldehyde reacts most strongly with nucleotides then proteins (Fraenkel-Conrat, 1954).

In vegetative cells, formaldehyde cytotoxicity has been linked to the formation of DNA-protein cross-links and single strand breaks. It has been recently demonstrated that DNA-protein cross-links are not directly involved in formaldehyde cytotoxicity in the yeast *Saccharomyces cerevisieae* (Sander and Brendel, 1988). The induction

of single strand breaks could still be a possible lethal mechanism as the frequency of these is known to increase with increasing formaldehyde concentration (Magna-schwenke *et al*, 1975; Poverenny *et al*, 1975). Hyperresistance of *E. coli* VU 3695 has been linked to a plasmid (94Kb). The plasmid codes for a protein, but the mechanism of action has not been elucidated. Hyperresistance of *E. coli* K12 and *Serratia marcescens* has also been linked to plasmids in the size range 58-90 mdal, the mechanism is thought to be linked to increased cell hydrophobicity (Kaulfers *et al*, 1987). Formaldehyde damage repair is related to U.V. repair in yeast (Magna-schwenke *et al*, 1978) and has at least one common step (Magna-schwenke and Ekert, 1978) suggesting that DNA-crosslinks are important.

1.9.2 Sporicidal Action

It has been suggested that the lethal action of formaldehyde on spores is due to an irreversible reaction involving nucleic acids (Trujillo and David, 1972). Its sporostatic action is thought to be due to an inhibition of germination. However, it may be that these early claims of sporicidal action could be due to sporostasis and not lethality as revival after killing by aqueous formaldehyde has been demonstrated (Spicher and Peters, 1976; 1981).

1.10 DEVELOPMENT OF LTSF

In 1961, Alder and Gillespie reported the successful disinfection of woollen blankets by the use of formaldehyde gas and steam at sub-atmospheric pressure below 100°C. Low Temperature Steam and Formaldehyde (LTSF) was not used in the NHS until later in the 1960's, when Alder *et al* (1966) adopted it as an alternative to

ethylene oxide sterilization after demonstrating the sporicidal activity of the system described in 1961. The system used in these first practical applications consisted of an evacuated chamber into which heated formaldehyde vapour followed by sub-atmospheric steam (LTS) were injected. Air was evacuated from the chamber at the start of the cycle. In a subsequent modification the air was removed by alternately evacuating and injecting small amounts of formaldehyde and LTS (Alder, 1968; Mitchell and Alder, 1970). This pulsed system was adapted by other centres for processing a wide range of objects and materials. Alder *et al*, (1971) reported the successful use of the formaldehyde pulsing system, with a final concentration of 8 ml/ft^3 formaldehyde and a temperature of 80°C. At about this time, 73°C was decided upon as the standard temperature (Gibson, 1982), though Weymes (1975) reported the use of temperatures as low as 65°C. The use of such low temperatures, and particularly any reduction below 65°C was advised against (Pickerill, 1975), as this would approach the thermal death point of many vegetative organisms. This would reduce the safety factor of killing any vegetative organisms by the temperature alone. Weymes and Harris (1980) reported that a very low failure rate in sterilizing spores of *B. stearothermophilus* was obtained (0.3%) using small pulses of LTS and formaldehyde over a long period. This appears to make a case for LTSF being a practical alternative to ethylene oxide for routine low temperature sterilization. Hurrell (1980) proposed that the most important requirement for successful sterilization was to obtain an homogenous mixture of monomeric formaldehyde gas and saturated steam. Other workers have stated that the maintenance of a low RH was a very important factor in the success of sterilization with LTSF (Gibson, 1982; Marcos and Wiseman, 1979).

LTSF as described here is not the only method of combining steam and formaldehyde that has been suggested for sterilization. Alder (1987) described a modification of the normal LTSF cycle in which dry formaldehyde gas was first injected followed by unsaturated water vapour. Saturated water vapour is then used to remove the formaldehyde. This process is known as FLTS. A similar exposure to formaldehyde, followed after a lag by LTS, was also found to be effective by Deverill and Cripps (1981).

Despite all these efforts to investigate the effect of formaldehyde and steam as a sterilizing process, very few LTSF machines are used as sterilizers (Gibson, 1982). This is probably due to controversy over the nature and control of the cycle, i.e. how and when formaldehyde is introduced and how spores for monitoring the process are presented (CSC Report, 1986).

1.11 PRINCIPLES OF LTS,LTSF AND FLTS

The principle of LTS is relatively simple. Steam is admitted to a previously evacuated chamber, and the temperature is adjusted to suit the items being processed. This can be achieved by varying the sub-atmospheric pressure of the steam to achieve the correct temperature. LTS has more disinfective power than water at the same temperature, due to the latent heat it possesses. When the steam condenses on the object, it gives up this latent heat to become water at the same temperature. Table 1.2 shows that the latent heat is a considerable proportion of the energy of the steam in relation to its sensible energy. LTSF and FLTS both possess this same latent heat capability, but combine it with the bactericidal/sporicidal properties of formaldehyde at raised temperatures. It has been proposed that it is

Table 1.2 Sensible and Latent Heat Available in Low Temperature Steam Between 60.06°C and 83.72°C

Pressure Bar Absolute	Temperature (°C)	Water Sensible Heat (KJ/Kg)	Evaporation Latent Heat (KJ/Kg)	Steam Total Heat (KJ/KG)
0.20	60.06	251.40	2358.3	2609.7
0.25	64.97	271.93	2346.3	2618.2
0.30	69.10	289.23	2336.1	2625.3
0.35	72.70	304.30	2327.2	2631.5
0.40	75.87	317.58	2319.2	2636.8
0.45	78.70	329.67	2312.0	2641.7
0.50	81.33	340.49	2305.4	2645.9
0.55	83.72	350.54	2299.3	2649.8

the synergistic effect of the properties of monomeric formaldehyde and LTS which gives LTSF its antimicrobial effect.

1.12 OPERATING CYCLES OF LTSF AND FLTS

1.12.1 LTSF

A typical operating cycle of a commercial LTSF machine is shown in Figure 1.6.

A pre-cycle warm up is often run to prevent condensation of the steam in a cool chamber. This usually consists of a short LTS cycle (not shown in Figure 1.6). The load is then put into the machine and the LTSF cycle is started. the typical cycle details illustrated in Figure 1.6 are described below :

i) Pre-vacuum

A pre-vacuum is drawn by vacuum pump to a pressure below 50 mbar. This removes most of the air from the chamber.

ii) Initial Steam Flush

A steam flush is included on many machines to allow the chamber to warm up to the operating temperature. The vacuum pump continues to operate during this part of the cycle.

iii) Pulsing

This stage can be divided into four components :

a) Steam is introduced until the temperature of 73°C is attained.

b) Vacuum is drawn to below 50 mbar.

c) Formaldehyde vapour (generated from formalin) is drawn into the chamber to give a final concentration of 15 g/m^3.

d) Optional Hold period to allow equilibration of gases.

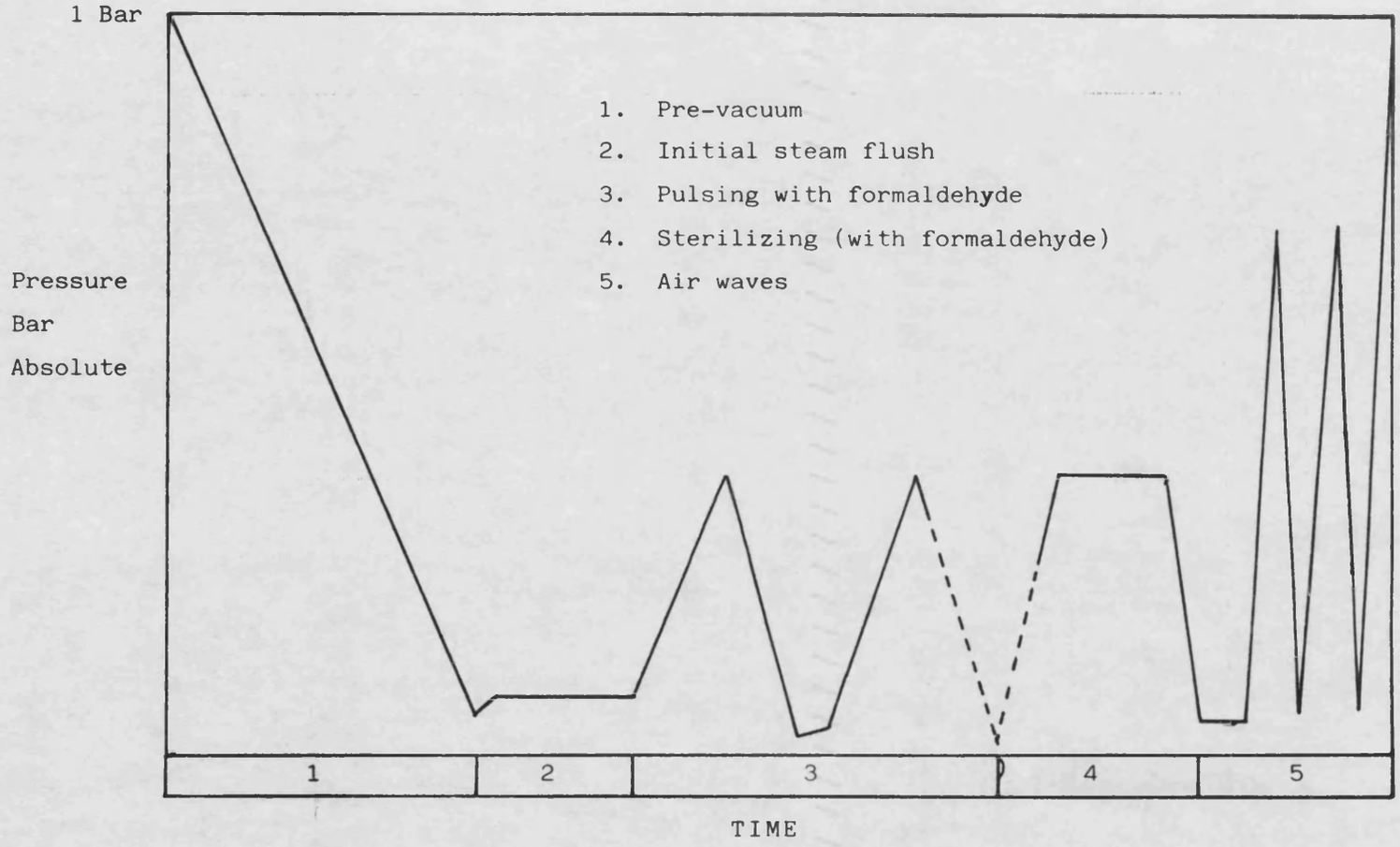


Figure 1.6 A 'Typical' Operating Cycle for an LTSF Sterilizer (from CSC Report, 1986)

e) Steam is admitted again as in a).

This pulsing may continue for up to 20 pulses.

iv) Final Steam Flush.

A series of alternating steam bleeds and vacuum pulses are used (similar to stage ii) to remove the formaldehyde.

v) Air Waves.

To remove the last traces of formaldehyde, a series of air pulses may be used. The final one will return the chamber to atmospheric pressure.

This is by no means a standard cycle and different cycles are used in commercial machines from different manufacturers. Some of the more common cycles are illustrated in Figure 1.7.

1.12.2 FLTS

The FLTS process as described by Alder (1987), depends upon initial formaldehyde adsorption onto the spore surface. The addition of the saturated steam at a later stage raises the temperature to 73°C and the relative humidity to 60-90%, which is considered optimal (Topley and Wilson, 1983). The reaction of the water molecules at this temperature then promotes a fast reaction between protein, formaldehyde and water. Latent heat does not play a part in this process as in LTS and LTSF (Alder, 1987). An illustration of the operating cycle is shown in Figure 1.8. A description of the stages is given below :

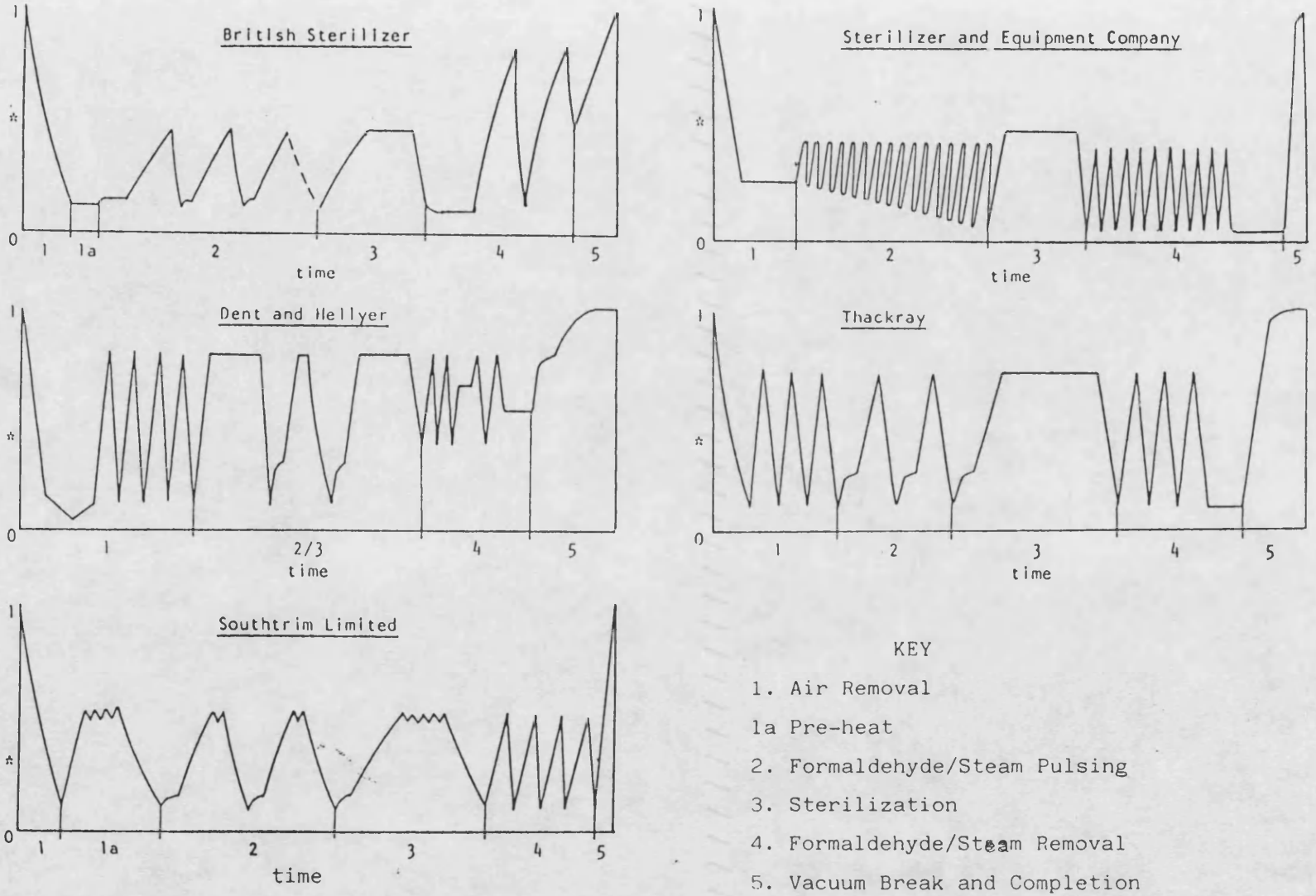


Figure 1.7 Range of LTSF Cycles used in Machines from Different Manufacturers (CSC Report, 1986)

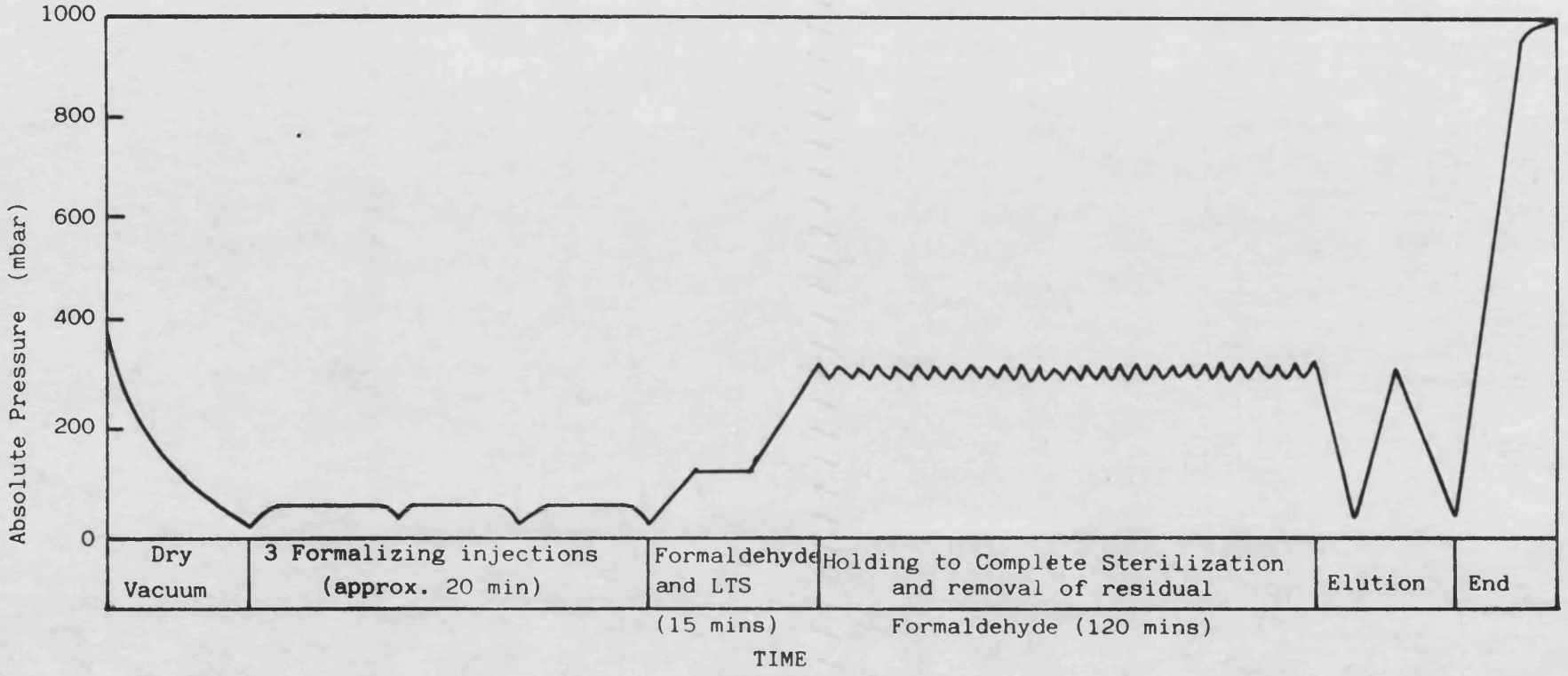


Figure 1.8 Operating Cycle of a FLTS Sterilizer (from Alder, 1987)

i) Dry evacuation of the chamber is carried out initially. The jacket is electrically heated to 1-5°C above the operating temperature to prevent condensation or superheat.

ii) Dry formaldehyde gas is generated in a heat exchanger (74 ml of formalin is vaporised per litre of chamber). This is introduced in three separate injections with three minute intervals.

iii) Again formaldehyde gas from the heat exchanger (8 ml/ft^3) is slowly injected into the chamber. When the pressure has stabilized (approx. 3 min), LTS is slowly introduced until the required operating conditions are achieved.

iv) Steam is continually injected for 120 minutes, completing the sterilization stage and removing formaldehyde from the chamber *via* the drain.

v) Evacuation stage, further residual formaldehyde is removed by use of alternating vacuum/steam pulses, with a minimum of two pulses.

vi) Aeration. Filtered air is introduced into the chamber to return it to atmospheric pressure.

Alder claims that in trials of this process, sterilization was achieved early in stage iv) of this cycle.

1.13 PROCESS VARIABLES AND THEIR CONTROL.

1.13.1 Temperature

The use of LTSF for the processing of heat labile items depends

upon the use of low operating temperatures to prevent damage to the load. However, the fact that formaldehyde polymerizes below 80°C must also be taken into account, lest all the formaldehyde be lost from the atmosphere. Heated jackets and doors on LTSF sterilizers, set at 1-5°C above the operating temperature enables temperatures below 80°C to be used. Although the use of temperatures between 65 and 80°C has been reported (Alder *et al*, 1971; Gibson. 1982), the accepted temperature for LTSF sterilization in the U.K. is 73°C ± 2°C (Gibson, 1982; CSC Report, 1986). This temperature appears to have been chosen solely on the basis that reproducible and successful cycles were obtained in a modified LTSF apparatus at this temperature (Alder, 1988). The increasing use in operating theatres of very thermolabile materials such as electronic apparatus requires the use of lower operating temperatures (Line and Cutts, 1983), in the region of 55°C.

1.13.2 Formaldehyde Concentration.

Hoxey (1984) demonstrated that an increase in concentration of formaldehyde from 6 mg/l to 27 mg/l led to increasing inactivation of spores of *B. stearothermophilus* NCIB 8224 spores at 80°C demonstrating the importance of formaldehyde concentration in LTSF.

Concentrations of formaldehyde between 3.3 mg/l and 100 mg/l have been used in the commercial LTSF sterilizers (Soper, 1988). In the U.K. the recommended formaldehyde concentration for LTSF sterilization is 25 mg/l. Handlos, 1979 reported that the level of residual formaldehyde in processed articles is related to the concentration used during sterilization and therefore recommended that the lowest concentration that is effective is desirable. These findings have subsequently been challenged. Hennebert (1987),

working on a general diffusion equation for formaldehyde, demonstrated that short sterilization periods with higher gas concentrations and temperatures left lower residuals. This appears to be true for at least 8 common polymers that were tested (Hennebert, 1988). It therefore appears that formaldehyde concentration and temperature may have to be optimised for each type of material to be sterilized. It is also possible that enhanced aeration, such as that demonstrated by Matthews *et al*, (1989) could reduce residual formaldehyde levels, allowing the use of longer LTSF cycles at lower operating temperatures.

1.13.3 Relative Humidity.

It has been demonstrated that a high relative humidity is required for efficient LTSF sterilization. Values of 70-90% (Alder, 1987) and 75-100% (Christensen and Kristensen, 1982) have been reported to be effective. The relative humidity must be strictly controlled, not only to maintain optimum conditions, but also to prevent condensation which would lead to formaldehyde polymerization. Relative humidity has also been shown to be an important factor in ethylene oxide sterilization (Dadd and Daley, 1980) and ozone sterilization (Ishizaki *et al*, 1986).

1.13.4 Vacuum

A high vacuum is required during LTSF to allow penetration of the formaldehyde into inaccessible parts of the load (such as narrow bore tubing). This is necessary due to the poor penetrating capability of formaldehyde as compared to such agents as ethylene oxide (Christensen and Kristensen, 1982). The sub-atmospheric pressures

used also allow pressure control of the steam temperature. Commercial LTSF machines usually operate at between 100 and 400 mbar, with the recommended value being 350 ± 30 mbar. Early cycle failures were associated with poor penetration of narrow bore tubing (Alder *et al*, 1966; Alder, 1968). For this reason, the design of test pieces for biological monitoring of sterilization must be designed to simulate these conditions as to prevent misleading results (Spichers and Bouchers, 1983).

1.13.5 Exposure Time

The recommended time for exposure of items in the U.K. is 2 h. This is shorter than that for ethylene oxide. These times may be shorter or longer according to the nature of the load, and the capacity of LTSF apparatus (Alder *et al*, 1971; Christensen and Kristensen, 1982). The residual concentrations left in the load must also be taken into account when choosing an exposure time (Hennebert, 1987; 1988).

CHAPTER 2.

GENERAL MATERIALS AND METHODS

2.1 APPARATUS

2.1.1 Glassware

Test tubes were rimless, 150 mm x 16 mm, of Pyrex glass with aluminium caps (Oxoid, London). Medical flat bottles of 500, 100 and 50 ml volume (FSA, Loughborough) with polypropylene caps were used throughout this work. All volumetric glassware was of grade B standard (FSA). Narrow neck, 250 ml, Erlenmeyer flasks were used for growth of microorganisms in liquid media. Glass universals of 25 ml volume were used for production of Nutrient Agar slopes. Glass spreaders were prepared from 3 mm glass, bent at an angle of around 60° to give approximately a 70 mm handle and a 30 mm spreading portion.

For sonication of test pieces (section 4.3.2), 30 ml thin glass screw top bottles (Aimer Products Ltd, London) were used.

2.1.2 Cleaning and Sterilization of Glassware

Glassware was soaked in Linkdet 704 (Link Chemicals Ltd), rinsed in tap water several times and twice in glass distilled water. Items were then dried in an oven at 160°C (Griffin 200FC). The openings of glassware were covered with aluminium foil. Spreaders were put into 250 ml beakers and the tops covered with foil. All glassware was sterilized in a hot air oven at 160°C (Griffin 200FC) for a minimum of 90 minutes.

2.1.3 Disposable Plasticware

Pre-sterilized single vent 10 cm polystyrene petri-dishes (Sterilin Products Ltd, Feltham) were used generally for growth, sporulation and recovery of microorganisms on solid media. Pre-

sterile polystyrene centrifuge tubes of 30 ml volume (Sterilin Products Ltd) were used for centrifugation and storage of liquid cultures and spore suspensions.

2.1.4 pH Measurement

The pH was measured using a Philips pH meter (Model PW 9418) with a Pye Unicam probe. Standardised calibration was carried out using pH buffer solutions of pH 4.0, 7.0 and 9.2, prepared from buffer tablets (FSA.).

2.1.5 Electrical Measurement

Measurements of voltage, current and resistance during modification of the Miniclave apparatus were carried out using a digital multimeter (Sinclair Electronics Ltd, St Ives).

2.1.6 Microscope and Objectives

A phase contrast microscope (Wild, Switzerland) fitted with X10, X40 and X100(oil) objectives (Wild, Switzerland) was used throughout this work.

2.1.7 Balances

Weights below 2 g were measured using an Oertling series 040 four digit balance and amounts above 2 g using an Oerling TP31 top pan balance.

2.1.8 Semi-Automatic Pipettes

The following pipettes were used,

1. Gilson model P5000 for volumes greater than 1 ml.
2. Gilson model P1000 for volumes between 0.2 ml and 1 ml.
3. Gilson model P200 for volumes less than 0.2 ml.

These pipettes were calibrated gravimetrically using distilled water at 25°C and an Oertling model series 040 balance. The atmosphere of the balance was humidified overnight by leaving an open petri-dish containing distilled water in the balance cabinet. Ten pipette volumes of water were pipetted into a weighing boat, each volume being weighed. The volumes chosen for each pipette were relevant to those for which the pipette would normally be used. To calculate the actual volume of water measured, 1 ml of water was taken to weigh 0.99007 g at 25°C (CRC Handbook of Chemistry and Physics, 1987-88). The coefficient of variation and the difference from nominal volume (that to which the pipette was set) were calculated. The results are recorded in Appendix 1.

2.1.9 Pipette Tips

All pipette tips were obtained from Anachem Ltd. Prior to use the tips were soaked in a dilute solution of Linkdet 704 (Link Chemicals Ltd), washed twice in tap water and several times in distilled water, and dried in a warm oven. These were then packed into autoclave bags (DRG Ltd, Bristol), heat sealed and autoclaved in a bench autoclave (Taylor stainless steel fittings Ltd, Leeds) for 15 minutes at 121°C.

2.2 MATERIALS

2.2.1 Bacterial Cultures

All bacterial strains used in this study were *Bacillus stearothermophilus* obtained from several International Type Culture

Collections, and these are listed below.

American Type Culture Collection (ATCC)

10149, 12016, 15951, 15952, 21365 and 29609.

National Collection of Industrial Bacteria (NCIB)

8222, 8224, 8920 and 8922.

Deutsche Sammlung von Mikroorganismen (DSM)

456, 457, 458, 1550, 2334 and 2349.

2.2.2 Preparation of Complex Media

2.2.2.1 Nutrient Agar (NA)

Nutrient Agar was used throughout this study as a growth media for maintenance of cultures, and for recovery of organisms after treatment with aqueous and gaseous formaldehyde. It was used in preference to Tryptone Soy Agar (TSA) as it gives smaller, more discrete colonies, and hence enumeration of colony forming units is made easier. Lab M Nutrient Agar powder was used. To prepare the medium 28 g of agar powder was dissolved in 1 litre of distilled water and volumes of approximately 400 ml dispensed into 500 ml medical flat bottles. The bottles were autoclaved at 121°C for 15 minutes in a bench autoclave. After cooling the agar to just over "hand hot" (50-60°C), approximately 15 ml aliquots were dispensed into petri-dishes. The agar was then allowed to cool and then stored inverted in the dark at for a maximum of one month at 4°C. Prior to use, the surface was overdried inverted at 37°C for 45 minutes.

2.2.2.2 Tryptone Soy Broth (TSB)

Oxoid Tryptone Soy Broth (TSB) was used for recovery of spores from liquid nitrogen and for growth of 18 hour cultures for inoculation of sporulation media. To prepare TSB 30 g of powder was dissolved in 1 litre of distilled water, and then dispensed in 100 ml volumes into 100 ml medical flat bottles. The medium was sterilized by autoclaving for 15 minutes at 121°C in a bench autoclave. Bottles of sterile TSB were stored for up to 3 months at room temperature in the dark.

2.2.3 Chemicals and Reagents

All chemicals used in this study were of Analytical Reagent (AR) grade and supplied from Sigma Chemical Co. (Poole, England), Aldrich (Gillingham, England) or B.D.H. Ltd (London, England) All vitamins were supplied by Sigma Chemicals and were stored at -20°C in the dark.

2.2.4 Water

All water for routine microbial use was glass distilled. Sterilization of water was carried out in 500 ml medical flat bottles. The bottles containing the water were sterilized at 121°C for 15 minutes in a bench autoclave. When preparing chemically defined media and solutions, water was boiled vigorously for 15 minutes, and then cooled rapidly to remove CO_2, immediately before use.

For washing and storage of spores, sterile double distilled deionised water was used to prevent clumping.

2.3 GENERAL METHODS

2.3.1 Microbiological Methods

2.3.1.1 Revival and Maintenance of Cultures

All bacteria were supplied by the Culture collections as freeze dried spores in glass ampoules. These spores were revived by inoculating them into TSB medium, and incubating for 18-24 hours at 56°C. This culture was used to inoculate Nutrient Agar plates and slopes. The remainder of the culture was left to settle and later aliquots of the cells were frozen in liquid nitrogen. The plates and slopes were incubated overnight and then taped up and stored at 4°C in the dark. The stored cultures were subcultured every 4 weeks.

2.3.1.2 Determination of Total Count

It has been demonstrated by Cook and Lund (1962) that haemocytometer slides of 0.1 mm depth with improved Neubauer ruling result in a lower estimate of total count than slides of depth 0.2 mm. However comparison of the accuracy of the estimates showed the 0.1 mm slides to be more accurate. For this reason a 0.1 mm depth slide with improved Neubauer ruling (Hawkey Ltd) was used in this study.

The coverslip was placed on the haemocytometer, if Newton's rings were not visible (rainbow colours) it was removed and the slide and coverslip cleaned with alcohol and the coverslip replaced. A suitable dilution of the spore suspension was prepared, to give 6-14 spores per small square, and a drop was allowed to be drawn under the coverslip by capillary action. The slide was then allowed to settle for 10 minutes. The slide was observed under a x40 phase contrast objective (Wild, Switzerland), and the number of phase

bright spores in 80 of the small squares of the counting chamber were recorded. The volume of one small square was 1/400 mm^2 x 0.1 mm depth. Therefore to calculate the total count, the average of the counts for the 80 squares observed was calculated, and this value multiplied by 4 x 10^6 to give the number of spores per cm^3 in the dilution of spore suspension that was used. Five replicate determinations were made. An example data set for *B. stearothermophilus* NCIB 8224 sporulated on Carbon limited medium is shown in Table 2.1.

Table 2.2 Quintuplicate Total Count Determinations for Spores of *B. stearothermophilus* NCIB 8224 sporulated on C-limited Medium. Dilution 10^{-1}

Sample	Mean of 80 Counts	Spores ml^{-1}	Mean
1	8.34	3.34 x 10^8	
2	8.93	3.57 x 10^8	
3	8.57	3.43 x 10^8	3.42 x 10^8
4	8.21	3.28 x 10^8	
5	8.71	3.48 x 10^8	

Standard deviation = 1.14 x 10^7

$$\text{Coefficient of Variation} = \frac{1.14 \times 10^7}{3.42 \times 10^8} \times \frac{100}{1} = 3.34\%$$

A coefficient of variation of 3.5% shows that the technique

was very reproducible with an acceptably small error, and was suitable for use in this study.

2.3.1.3 Determination of Viable Counts

The spread plate technique was used to ascertain the number of viable organisms in a spore suspension, (i.e. the viable count). Suitable 10 fold dilutions (1 ml + 9 ml) of the spore suspensions were carried out in sterile double distilled deionised water to give a final suspension containing between 200 and 750 spores/ml. Before each dilution, the suspension was vortexed on a whirlimixer (Fisons). 0.2 ml samples of the final suspension were spread onto the surface of five replicate Nutrient Agar plates using a glass spreader. These were left to stand for 15 min to allow the 0.2 ml to absorb, and the plates were then incubated inverted at 56°C +/- 1°C for five days, until single colonies (1-4 mm diameter) were visible. The number of colonies were then recorded. An example of a viable count determination of batch A26 (NCIB 8224 spores sporulated on C-LTD medium) is shown in Table 2.2. The coefficient of variation of 3.0% indicates that the method is suitable for use in this study.

2.3.2 Chemical Methods

2.3.2.1 Assay of Formaldehyde

Sodium Sulphite Assay

Every bottle of formalin supplied by Aldrich Chemicals was stated to be 38% w/v formaldehyde with 15% methanol as a stabiliser. The Sodium Sulphite method was used to confirm the concentration of formaldehyde, in all solutions of formalin prior

Table 2.2 Quintiplicate Determination of the Viable Count of Spores of *B. stearothermophilus* NCIB 8224 (BA26) recovered on Nutrient Agar Plates incubated at 56°C for 5 days

Sample	Colony counts per plate	Mean count	Mean Viable
1	142 148 153 145 140		
2	160 154 148 149 157		
3	141 145 148 143 150	148.32	7.416×10^7
4	149 151 148 143 150		
5	148 152 154 142 145		

Analysis of Variance

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Between Samples	4	203.44	50.86	2.6
Within Samples	4	94.64	23.66	1.2
Residual	16	307.36	19.21	
Total	24	605.44		

$F(4,16)\ P_{0.05} = 3.01$

Coefficient of Variance = 3.0%

to use.

A 50 ml volume of a molar solution of sodium sulphite (AR grade Aldrich) and three drops of a 0.1% alcohol solution of thymolphthalein indicator were added to a 500 ml Erlenmeyer flask. This mixture was carefully titrated with normal sulphuric acid until the blue colour disappeared (usually 3 drops). A volume of formalin was carefully weighed (approx. 3 g) and added to the flask. A blue colouration developed, and this was titrated slowly with the acid until the blue colour was once again neutralized. The percentage of formaldehyde in the mixture was then calculated by :

$$\% \text{ formaldehyde} = \frac{\text{Acid titre} \times \text{Normality of acid} \times 3.003}{\text{Weight of sample}}$$

3-Methylbenzothiazolinone Hydrazone (MBTH) Assay.

A calibration curve was constructed using appropriate aqueous dilutions of a 38% formalin solution were carried out to give a range of formaldehyde concentrations between 0 and 4 µg/ml. A 0.1% w/v aqueous solution of MBTH (Aldrich Co. Ltd.), and an oxidising solution containing 1.6% w/v sulphamic acid and 1.0% w/v ferric chloride were also prepared.

Aliquots (5 ml) of formaldehyde solution and MBTH solution were then mixed in a test tube to give a final volume of 10 ml. These solutions were then vortexed and left to stand for 60 min at 25°C. After standing, 2 ml of the oxidising solution was added to each of the tubes, vortexed and left to stand for a further 12 min, to allow the blue colour to fully develop.

Using a spectrophotometer (Milton Roy, 601), the absorbance of each solution was measured at a wavelength of 628 nm. A calibration plot was constructed by plotting the absorbance (ordinate) against the formaldehyde concentration (abcissa) (Figure 2.1). The plot obeys Beer's law between 0 and 1.9 ug/ml concentrations of formaldehyde. As the relationship has been demonstrated to be linear, it was possible to calculate the Molar Extinction Coefficient (E). This is related to the absorbance and formaldehyde concentration by the equation (Fell, 1986)

$$E = \frac{A}{C\ L}$$

E = Molar Extinction Coefficient (Litre/mol/cm)

A = Absorbance

L = Path length (cm)

C = Concentration (Moles/l)

The Molar Extinction Coefficient for formaldehyde measured by MBTH at 628 nm, calculated from the absorbance at 0.95 µg/ml concentration (Figure 2.1) is therefore :

$$E = \frac{0.93}{3.15 \times 10\text{-}5 \times 1}$$

E = 29523.8 litre/mol/cm

This figure for E can now be used to calculate the concentration of formaldehyde from absorbance data, by transposing the equation used to calculate E.

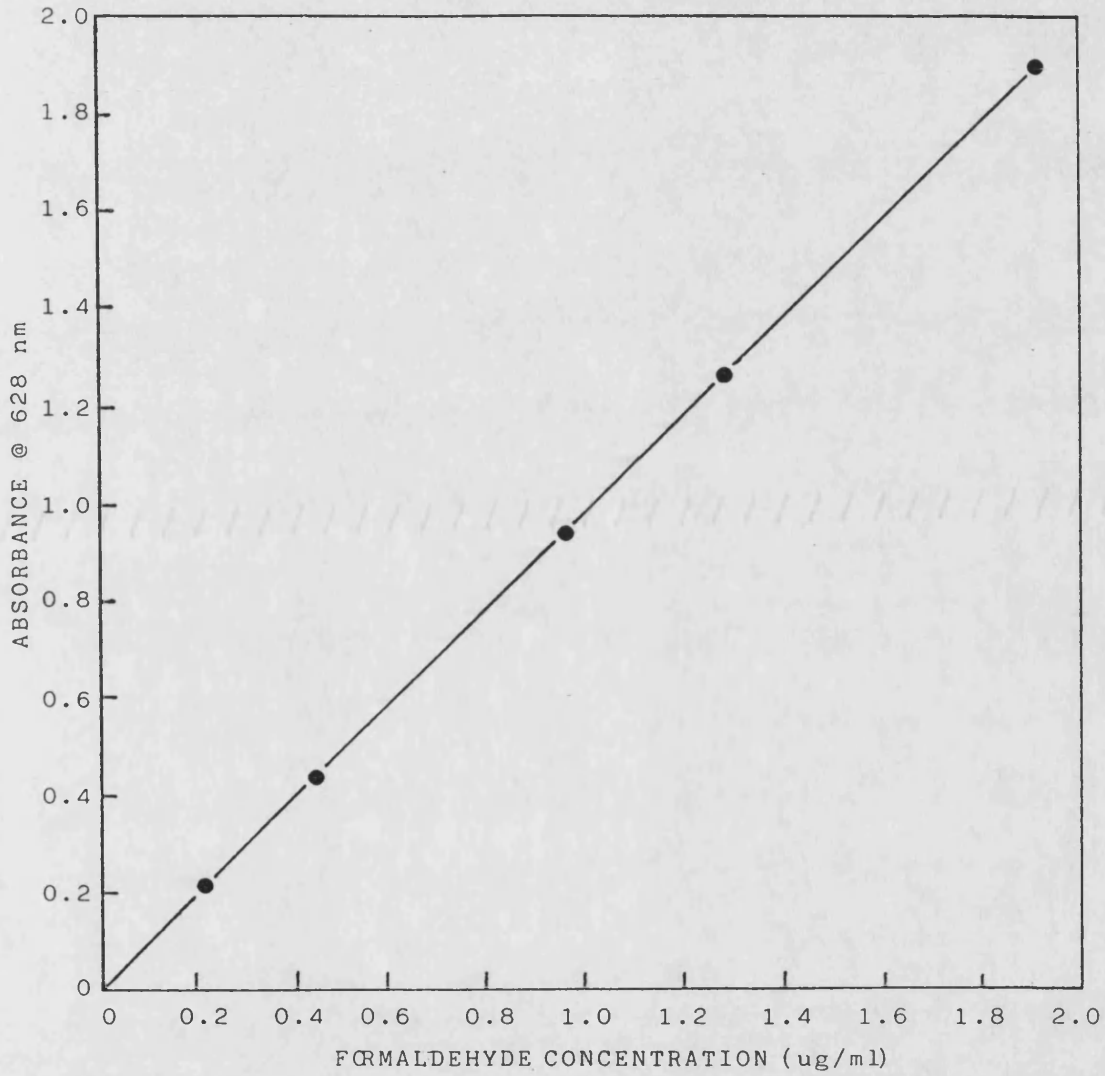


Figure 2.1 Calibration Graph for the MBTH Spectrophotometric Assay for Formaldehyde

CHAPTER 3

GROWTH, SPORULATION AND CHARACTERISATION OF 16 STRAINS OF BACILLUS STEAROTHERMOPHILUS ON DEFINED MEDIA

3.1 INTRODUCTION

3.1.1 Production of Spores

Of the ideal properties of a biological indicator organism, discussed in Chapter 1, the most important is that the organism should produce spores with predictable and reproducible inactivation characteristics.

Spores of *Bacillus* spp. have been used successfully for the monitoring of several sterilization processes. These include *Bacillus subtilis* var. *niger* spores for monitoring ethylene oxide sterilization (Dadd and Daley, 1980) and *Bacillus stearothermophilus* for steam sterilization processes (Kelsey, 1961). *B. stearothermophilus* has also been considered as a potential indicator for ultraviolet radiation sterilization (Abshire *et al*, 1983). At present there is no monitor available specifically for LTSF sterilization, and using the strains chosen for other sterilization processes has proved unsuccessful. The heat used in LTSF would rapidly kill the *B. subtilis* spores used for ethylene oxide monitoring (Hoxey *et al*, 1985), and the use in LTSF sterilization of *B. stearothermophilus* NCTC 10003 spores that were used originally for autoclave monitoring, has proven unreliable (Cripps *et al*, 1976).

The resistance characteristics of spores are determined by three major factors; 1) genetic, 2) conditions during growth and sporulation 3) conditions during recovery. The genetic factors can be taken into account by use of organisms whose spores have inherently high heat resistance, e.g. *B. stearothermophilus*. Screening a wide range of strains of this organism might identify an organism with near ideal characteristics for a biological indicator

organism for LTSF. One of the important factors during growth and sporulation which influences spore resistance is the media composition. It has been demonstrated that it is possible to manipulate predictably the resistance characteristics of spores by altering the chemical composition of the medium on which they are sporulated (Lee and Brown, 1975). This can lead to the production of spore crops with higher and more reproducible resistance and germination characteristics (Hodges *et al*, 1980; Jones and Pflug, 1981), or modified spore characteristics (Brown and Hodges, 1974; Lee and Brown, 1975).

Chemically defined media have been used successfully by several workers to produce high yields of spores with desirable inactivation characteristics (Hoxey, 1984; Hurrell, 1988; Chinyanganya, 1989). The media used in this study are some of the more successful ones used for the production of *Bacillus stearothermophilus* spores by these workers.

1) SSMAVIT medium is used in both solid and liquid form. It was first described (in its present form) by Hobbs (1975), who developed it from a medium described by Lazarini and Santangelo (1967). This was used successfully by both Hoxey (1984) and Chinyanganya (1989). Though this is a chemically defined medium, it is the most complex of the media used here in terms of the number of components.

2) De Guzmans medium is used only in solid form. This was developed by De Guzman (1972) from a medium described by Campbell and Williams (1953a and b) for the sporulation of the smooth (s) variant of *B. stearothermophilus* NCA 1518.

3) Andersons medium is used only as a liquid. This was developed by

Anderson and Friesen (1972) to sporulate *B. stearothermophilus* NCTC 10003 used for monitoring steam sterilization cycles.

4) Carbon limited (C-Ltd) medium is used only in solid form. It was first developed by Steele (1987) from a medium described by Lee and Brown (1975). It is a relatively simple medium, and is currently under investigation as a medium for producing spores of *B. stearothermophilus* NCTC 10003, at the Dept. of Health LTSF Reference Laboratory at Luton College.

3.1.2 Spore Harvesting and Cleaning

For a spore crop to be characterised as to yield, growth index and inactivation kinetics it is important for the crop to be free of any vegetative cells and debris (Waites and Bayliss, 1979). The presence of vegetative cells would give an artificially high viable count, and would result in a rapid drop in the surviving fraction in the first few minutes of an inactivation experiment at high temperatures, i.e. a type C curve. Vegetative debris could also interfere in resistance studies by coating and hence protecting the spores from the inactivating conditions (This could, for example, result in reduced diffusion of formaldehyde into the spore).

Various methods have been proposed and used to produce clean spore suspensions. The first of these methods is used to facilitate the release of spores from the sporangia, and involves treating the sporangia with lysozyme (Finley and Fields, 1962) or sodium sulphate (Burnett *et al*, 1986). These methods would be considered unacceptable for the production of spores for use as Biological Monitors as they involve the use of chemical treatments which may have an undesirable effect on the spore characteristics. Other

methods have been used to free spores from sporangia, such as sonication or heating (Phillips and Martin, 1982). These should be avoided if possible, as sonication has been reported to have a deleterious effect on heat resistance of spores (Burgos *et al*, 1972), and heating could have other, as yet undefined, effects.

Once all of the spores are in free suspension, they must be separated from the debris. The methods to do this include two phase separation (Sachs, 1969), which relies on partitioning spores and debris between polyethylene glycol and phosphate buffer; and density gradient centrifugation (DGC) (Prentice *et al*, 1972). The drawbacks of these are that DGC is complex and time consuming, and both methods involve chemical treatment with possibilities for undesirable effects.

On the basis of the potential drawbacks of all these methods, it was decided to avoid using any method to increase release of spores from sporangia, and to use a purely physical method for separation of spores and debris. The method of centrifugation as described by Long and Williams (1958) and used successfully by Hoxey (1984) and Chinyanganya (1989) was chosen.

3.1.3 Characterisation of Spore Crops

Once the spore crops had been produced, it was necessary to characterise them so that comparison of the different spore batches could be carried out. From this it was intended to choose two organisms for further study, one primary and one backup in case the first was shown to be unsuitable. The characteristics by which they were compared were in part chosen to reflect some of the ideal properties of an indicator organism. These included ease of growth, percentage sporulation, ease of cleaning, growth index, resistance

to formaldehyde and type of survivor curve.

The most obvious way to test batches of spores for suitability as a monitor organism would be to expose them to LTSF test conditions over various time periods and then compare the survivor curves (Chapter 6). This was not possible as no working apparatus existed that was capable of allowing introduction and removal of samples throughout an LTSF cycle. Therefore it was necessary for initial testing to employ an aqueous formaldehyde system as used by Hoxey (1984) and Chinyanganya (1989). This would not mimic the LTSF conditions, but was a practical way of comparing the resistance of various spore batches to both heat and formaldehyde. The temperature and the formaldehyde concentration were chosen to be close to those which the organism would need to survive under "typical" LTSF conditions. From these results it was considered possible to select the spores with the most desirable combination of resistance and survivor curve type to use in later experiments.

3.2 Materials and Methods.

3.2.1 Bacterial Strains

Sixteen strains of *Bacillus stearothermophilus* were used in this study. These are are listed in section 2.2.1.

3.2.2 Composition and Preparation of Defined Media

Bacillus stearothermophilus does not produce spores as readily as many of the other *Bacillus* spp. (Anderson and Friesen, 1972). Several media have been reported to produce adequate sporulation in this

species, and these are described in 3.1.1. SSMAVIT (in solid and liquid form), DeGuzmans (solid), Andersons (liquid) and Carbon LTD (solid) were used in this study and their composition and preparation are described in 3.2.2.1 to 3.2.2.4.

For all defined media, freshly boiled glass distilled water was used to dissolve the ingredients, using gentle heat only if necessary.

3.2.2.1 Composition and Preparation of Complete SSMAVIT medium.

The composition of complete SSMAVIT medium is shown in Table 3.1. The complete medium was prepared by the mixing of four sterile stock solutions under laminar flow. The compositions of the four stock solutions is shown in Table 3.2.

All of the ingredients for solution I were dissolved into 1.8 litres of ditilled water using gentle heat. The solution was cooled, the pH adjusted to 6.9-7.1 and the volume made up to 2 litres. The solution was then filter sterilized through a 100mm diameter 0.22 µm membrane using a Sartorius positive pressure filtration apparatus. All of this was carried out under laminar flow. The first 200mls of filtrate was discarded and the remaining solution stored as 200ml aliquots at 4°C in the dark.

Solutions of the compounds for solutions II and III were prepared, as indicated in Table 3.2. The solutions were then filter sterilized as for solution I, with the first 50 ml being discarded, and the remaining filtrate being stored in 25 ml aliquots at 4°C in the dark.

The vitamins for solution IV were dissolved in 450 ml of distilled water and the pH adjusted to 6.8 and then made up to volume. The solution was sterilized by positive pressure filtration, the first

Table 3.1 Composition of Complete SSMAVIT Medium

Constituent	Molarity	Amount (gl^{-1})
Potassium dihydrogen phosphate	1.76×10^{-2}	2.4
Dipotassium hydrogen phosphate	3.21×10^{-2}	5.6
Sodium citrate	3.20×10^{-3}	1.0
"TRIS" buffer base	2.98×10^{-2}	2.4
L-glutamic acid	3.02×10^{-2}	4.45
L-tryptophan	1.22×10^{-4}	0.025
L-methionine	1.68×10^{-4}	0.025
L-histidine HCl	4.51×10^{-4}	0.07
L-leucine	9.51×10^{-4}	0.12
L-valine	7.68×10^{-4}	.09
Ferrous sulphate ($7H_2O$)	1.00×10^{-5}	0.003
Magnesium chloride ($6H_2O$)	1.00×10^{-3}	0.203
Zinc sulphate ($7H_2O$)	5.00×10^{-5}	0.010
Calcium chloride ($2H_2O$)	1.00×10^{-3}	0.147
Manganese chloride ($4H_2O$)	5.00×10^{-5}	0.010
Thiamine HCl	4.45×10^{-7}	0.00015
Nicotinic acid	1.22×10^{-5}	0.0015
Biotin	4.09×10^{-8}	0.00001
Pyridoxal HCl	5.38×10^{-7}	0.00009
Folic acid	1.36×10^{-7}	0.00006
D-glucose	8.33×10^{-3}	1.5

TABLE 3.2 Preparation of Sterile Stock Solutions for SSMAVIT Medium

Solution	Component	Amount	Water to	Stored as
Solution I	K_2HPO_4	28.00 g	2000 ml	200 ml aliquots
	KH_2PO_4	12.00 g		
	Tri-sodium citrate	5.00 g		
	'Tris'buffer	18.00 g		
	L-glutamic acid	22.25 g		
	L-tryptophan	0.125 g		
	L-methionine	0.125 g		
	L-leucine	0.60 g		
	L-histidine	0.35 g		
	L-valine	0.45 g		
	D-glucose	7.50 g		
Solution II	$FeSO_4.7H_2O$	50 ml	250 ml	
	$MgCl_2.6H_2O$ (0.1 M)	50 ml		
	$ZnSO_4.7H_2O$ (0.01M)	25 ml		
Solution III	$CaCl_2.2H_2O$ (0.1M)	50 ml	250 ml	
	$MnCl_2.4H_2O$ (0.01M)	25 ml		
Solution IV	Thiamine HCl	0.030 g	500 ml	
	Nicotinic acid	0.300 g		
	Biotin	0.002 g		
	Folic acid	0.012 g		
	Pyridoxal HCl	0.018 g		

100 ml being discarded, and the rest sealed as 1.5 ml aliquots in 2 ml ampoules. These were stored as -20°C until needed. Agar solution was prepared by making a 250 ml of a 2.4% solution of Agar Technical No 3 (Oxoid). This was sterilized at 121°C for 15 minutes in 500 ml medical flat bottles.

The final medium was prepared by mixing these stock solutions in the proportions and order indicated in Table 3.3. The solutions were added to sterile water (for liquid media) and molten agar at 55°C (for solid media). The solutions were added very slowly with gentle mixing to prevent precipitation. Liquid media was dispensed in 50 ml volumes into 250 ml Erlenmeyer flasks and solid media into 90mm diameter petri-dishes.

Table 3.3 Preparation of Complete SSMAVIT Medium from Sterile Stock Solutions

Solution	Quantity (ml)
Solution I	200.00
Solution IV	1.25
Solution II	25.00
Solution III	25.00

3.2.2.2 Composition and Preparation of Complete De Guzmans Medium.

The composition of complete De Guzmans medium is shown in Table 3.4. As with SSMAVIT, it is prepared from sterile stock solutions, the compositions of which are described in Table 3.5.

The constituents of solution I were dissolved and the pH adjusted to 6.5 and the solution made up to volume. Sterilization

Table 3.4 Composition of Complete De Guzman's Medium

Constituent	Molarity	Amount (gl^{-1})
Potassium dihydrogen phosphate	7.35×10^{-3}	1.0
Disodium hydrogen phosphate	6.98×10^{-3}	2.4
Sodium acetate ($3H_2O$)	3.70×10^{-3}	0.504
Sodium chloride	1.70×10^{-3}	1.0
Ammonium chloride	1.87×10^{-2}	1.0
L-leucine	1.07×10^{-3}	0.14
Ferric chloride ($6H_2O$)	3.10×10^{-6}	0.00085
Magnesium chloride ($6H_2O$)	5.20×10^{-6}	0.00105
Calcium chloride ($2H_2O$)	4.50×10^{-6}	0.001
Manganese chloride ($4H_2O$)	5.05×10^{-4}	0.1
Thiamine HCl	4.45×10^{-7}	0.00015
Nicotinic acid	1.22×10^{-5}	0.0015
Biotin	4.09×10^{-8}	0.00001
Pyridoxal HCl	5.38×10^{-7}	0.00009
Folic acid	1.36×10^{-7}	0.00006
D-glucose	1.10×10^{-2}	2.0

Table 3.5 Preparation of Sterile Stock Solutions for De Guzman's Medium

Solution	Component	Weight (g)	Water to	Stored as
Solution I	L-leucine	0.175	500 ml	200 ml aliquots
	D-glucose	2.5		
	$CH_3COONa.3H_2O$	0.63		
	Na_2HPO_4	3.0		
	KH_2PO_4	1.25		
	NH_4PO_4	1.25		
	NaCl	1.25		
Solution II	$MgCl_2.6H_2O$	0.021	1000 ml	200 ml aliquots
	$FeCl_3.6H_2O$	0.017		
Solution III	$MnCl_2.4H_2O$	4.0	1000 ml	25 ml aliquots
	$CaCl_2$	0.02		
Solution IV	Thiamine HCl	0.03	500 ml	1.5 ml ampoules
	Nicotinic acid	0.3		
	Biotin	0.002		
	Pyridoxal HCl	0.018		
	Folic acid	0.012		

and storage were as for SSMAVIT solution I. The ingredients of solutions II and III were dissolved with gentle heating, cooled and made up to volume. Sterilization and storage were as for SSMAVIT II and III. Solution IV was prepared, and was the same composition as SSMAVIT solution IV.

The final medium was prepared by mixing these solutions in the order and proportions into molten agar as indicated in Table 3.6.

Table 3.6 Preparation of Complete Deguzman's Medium from Sterile Stock Solutions

Solution	Quantity (ml)
Solution I	400.00
Solution VI	2.50
Solution II	50.00
Solution III	25.00
Distilled water	22.50
Molten Agar Solution	500.00

3.2.2.3 Composition and Preparation of Complete Anderson's Medium.

The composition of this medium is shown in Table 3.7. As with the previous two media, it was prepared from sterile stock solutions. The compositions of these solutions are shown in Table 3.8. Each was prepared as for the corresponding De Guzman's solution.

The final medium was prepared by mixing these solutions in the order and proportions shown in Table 3.9, and distributed between

Table 3.7 Composition of Complete Anderson's Medium

Constituent	Molarity	Amount (gl^{-1})
D-glucose	1.00×10^{-3}	0.18
Ammonium hydrogen phosphate	1.00×10^{-2}	1.32
Potassium dihydrogen phosphate	1.00×10^{-2}	1.74
ammonium sulphate	1.00×10^{-2}	1.32
Magnesium sulphate ($7H_2O$)	5.00×10^{-4}	0.123
Ferrous sulphate ($7H_2O$)	1.00×10^{-5}	0.0028
Manganese sulphate ($4H_2O$)	1.00×10^{-4}	0.022
Calcium chloride ($2H_2O$)	1.00×10^{-4}	0.022
L-glutamic acid	2.40×10^{-3}	0.353

Table 3.8 Preparation of Sterile Stock Solutions for Anderson's Medium

Solution	Component	Weight (g)	Water to	Stored as
Solution I	D-glucose	0.9	200 ml	2000 ml aliquots
	L-glutamic acid	1.75		
	$(NH_4)2HPO_4$	6.6		
	$(NH_4)SO_4$	6.6		
	KH_2PO_4	8.7		
Solution II	$MgSO_4.7H_2O$	2.46	25 ml	1000 ml aliquots
	$FeSO_4.7H_2O$	0.056		
Solution II	$MnSO_4.4H_2O$	0.44	25 ml	1000 ml aliquots
	$CaCl_2.2H_2O$	0.44		

250 ml Erlenmeyer plastics.

Table 3.9 Preparation of Complete Anderson's Medium from Sterile Stock Solutions

Solution	Quantity (ml)
Solution I	200
Solution II	25
Solution III	25
Sterile Distilled water	250

3.2.2.4 Composition and Preparation of Complete Carbon LTD Medium.

The complete composition of this medium is shown in Table 3.10. As with the media described previously, it was prepared by aseptically mixing sterile stock solutions, the compositions of which are shown in Table 3.11.

The ingredients of solution I were disssolved and made up to volume. Sterilization was by positive pressure filtration as for previous media. This solution was stored as 100 ml aliquots at 4 °C in the dark.

Solution II (Sorensens phosphate buffer) was prepared by dissolving the ingredients in freshly boiled and cooled distilled water. Sterilization was by positive pressure filtration, and 100 ml aliquots were stored at 4°C were stored at 4°C in the dark.

Solution III, the electrolyte solution, was made up just prior to

Table 3.10 Composition of Complete Carbon Limited Medium

Constituent	Molarity	Amount (gl^{-1})
Glutamic acid	2.40×10^{-3}	0.353
Glucose	1.00×10^{-3}	0.180
Methionine	4.50×10^{-5}	7.0×10^{-3}
Magnesium chloride ($6H_2O$)	1.80×10^{-5}	3.7×10^{-3}
Manganese chloride ($4H_2O$)	1.50×10^{-5}	3.0×10^{-3}
Calcium chloride ($2H_2O$)	5.50×10^{-5}	8.1×10^{-3}
Ferrous sulphate ($7H_2O$)	1.00×10^{-5}	2.8×10^{-3}
Nicotinic acid (Na salt)	6.00×10^{-7}	9.0×10^{-5}
Thiamine HCl	2.50×10^{-8}	8.0×10^{-6}
Biotin	8.00×10^{-10}	2.0×10^{-7}
Disodium hydrogen phosphate	1.76×10^{-2}	2.498
Potassium dihydrogen phosphate	7.30×10^{-3}	0.993

Table 3.11 Preparation of Sterile Stock Solutions for Carbon Limited Medium

Solution	Component	Amount	Water to	Stored as
Solution I	Glutamic acid D-glucose L-methionine	0.450 g 0.180 g 0.007 g	100 ml	100 ml aliquots
Solution II	KH_2PO_4 Na_2HPO_4	0.993 g 2.498 g	100 ml	100 ml aliquots
Solution III	$MgCl_2.6H_2O$ $MnCl_2.4H_2O$ $CaCl_2.2H_2O$ $FeSO_4.7H_2O$	3.700 g 3.000 g 8.100 g 2.800 g	500 ml	Not stored
Solution IV	Thiamine HCl Soln. (0.8 g/l)	1.0 ml	100 ml	1.5ml ampoules
Solution V	Biotin Solution (0.2 g/l)	1.0 ml	1000 ml	1.5 ml ampoules
Solution VI	Nicotinic acid Soln. (0.9 g/l)	1.0 ml	10 ml	1.5 ml ampoules

preparation of the final medium. The reason for this is that the ferrous sulphate rapidly oxidises to ferric sulphate causing precipitation. The ingredients were dissolved (without heating), made up to volume, and filter sterilized as with previous solutions.

Solutions IV to VI are vitamin solutions. To measure the small amounts needed accurately, a series of dilutions were carried out as follows. Solution IV was prepared by dissolving 0.8 g of Thiamine HCl into 1 litre of distilled water, then 1 ml of this solution was added to 99 ml of distilled water to make solution IV. Solution V was prepared by dissolving 0.2g of Biotin in 1 litre of ditilled water, then 1 ml of this solution was added to 999 ml of distilled water to make solution V. Finally, solution VI was prepared by dissolving 0.9g of Nicotinic acid (sodium salt) into 1 litre of distilled water, the 1 ml of this added to 9 ml of distilled water to form the final solution.

To prepare the complete medium, the solutions were mixed in the order and proportions shown in table 3.12. Once the 3% agar solution has been added, the medium was dispensed into 90 mm petri-dishes and stored at 4°C in the dark for up to 4 weeks.

3.2.3 Preparation of Inocula.

Each of the test strains were inoculated into 100 ml of sterile Tryptone Soy Broth (TSB) contained in 250 ml Erlenmeyer flasks and incubated at 55°C for 18 h.

The 18 h cultures were dispensed into 30 ml sterile plastic centrifuge tubes (Sterilin), and centrifuged at 4000 rpm for 15 minutes in a MSE Chilspin centrifuge. The supernatants were discarded, and the pellets combined and resuspended in 20 ml of a liquid form of the defined medium to be inoculated. The

centrifugation and resuspension were repeated once more to ensure the removal of any remaining complex medium.

Table 3.12 Preparation of Complete Carbon Limited Medium from Sterile Stock Solutions

Solution		Quantity (ml)
Solution I		100.0
Solution II		100.0
Solution III		0.5
Solution IV		1.0
Solution V		1.0
Solution VI		1.0
Sterile Distilled Water	to	500.0
Sterile 3% Oxoid Tech. No 3 agar		500.0

3.2.4 Inoculation and Incubation of Defined Media

All but one of the strains of *B. stearothermophilus* listed in section 2.2.1 were inoculated and sporulated on each of the defined media. *B. stearothermophilus* NCIB 8224 was the exception, only being sporulated on C-Ltd medium. This was because sporulation of this organism on the other four media had previously been shown to be poor (Hoxey, 1984).

For the liquid media, 1 ml of the culture prepared in 3.2.3 was inoculated into a 50 ml volume of the media contained in a 250 ml Erlenmeyer flask. For solid media the plates were overdried at 37°C for 1 h. A 1 ml inoculum of the culture prepared in 3.2.3 was put

onto the surface of the plate, and spread using a sterile glass spreader. These plates were then left to stand for 15 min to absorb the liquid, then any excess liquid was removed carefully using a sterile pasteur pipette.

For a typical batch of spores five flasks and ten plates were inoculated with a test strain, though this was adjusted in several instances when growth was poor.

Flasks were incubated unshaken at 55°C in an incubator. Plates were inverted and incubated at 55°C. Samples of the cultures were taken every day, and examined using a phase contrast microscope. The amount of growth and the proportion of spores (in %) was estimated. If no increase in either of these was noted for three consecutive days, then maximum growth and sporulation was assumed to have been obtained. If less than 50% sporulation or very low growth occurred, then that combination of organism and medium was considered unsuccessful and abandoned. The results of these studies are illustrated in Tables 3.13 - 3.15.

3.2.5 Harvesting and Cleaning of Spore Crops

For "successful" combinations of organisms and media, the spores were harvested and cleaned. For cultures on solid media 5 ml of ice cold sterile double distilled deionised water (SDW) was pipetted onto the agar, and the spores suspended in the water by lifting them off the plate using a sterile glass spreader. The suspension was then pipetted into 30 ml plastic centrifuge tubes (Sterilin). For liquid media the media plus organisms was dispensed directly into the centrifuge tubes.

The suspensions were centrifuged at 4000 rpm at 4°C for 15 min in a MSE Chilspin to form a pellet. The supernatants were discarded,

Table 3.13 Extent of Growth and Sporulation of the ATCC Strains of *B.stearothermophilus* on Different Sporulation Media

ATCC Strain Number	Sporulation Medium	Growth	Sporulation (%)	Maximum by Day :
	S-SSMAVIT	++	<1	14
	L-SSMAVIT	++	<1	14
10149	De Guzman's	++++	0	14
	Anderson's	+	0	14
	Carbon LTD	++	<1	14
	S-SSMAVIT	++++	95	6
	L-SSMAVIT	+++	90	6
12016	De Guzman's	+++	1	14
	Anderson's	+	<1	14
	Carbon LTD	++	90	13
	S-SSMAVIT	++	15	14
	L-SSMAVIT	+++	<1	14
15951	De Guzman's	++	30	14
	Anderson's	+	2	14
	Carbon LTD	++	35	14
	S-SSMAVIT	++++	95	6
	L-SSMAVIT	++	90	6
15952	De Guzman's	+++	55	8
	Anderson's	++	90	4
	Carbon LTD	+++	40	14
	S-SSMAVIT	++++	95	3
	L-SSMAVIT	++	30	14
21365	De Guzman's	+++	90	6
	Anderson's	+	10	14
	Carbon LTD	++	80	6
	S-SSMAVIT	++	1	14
	L-SSMAVIT	++	0	14
29609	De Guzman's	+	1	14
	Anderson's	++	<5	14
	Carbon LTD	+	0	14

Table 3.14 Extent of Growth and Sporulation of the DSM Strains of *B.stearothermophilus* on Different Sporulation Media

DSM Strain Number	Sporulation Medium	Growth	Sporulation (%)	Maximum by Day :
	S-SSMAVIT	++	<1	14
	L-SSMAVIT	++	<1	14
456	De Guzman's	+++	5	14
	Anderson's	+	<1	14
	Carbon LTD	+	<5	14
	S-SSMAVIT	+	0	14
	L-SSMAVIT	+	0	14
457	De Guzman's	+	1	14
	Anderson's	+	<1	14
	Carbon LTD	+	1	14
	S-SSMAVIT	+++	60	11
	L-SSMAVIT	++	50	13
458	De Guzman's	+++	1	14
	Anderson's	+	1	14
	Carbon LTD	+	1	14
	S-SSMAVIT	+	2	14
	L-SSMAVIT	+	2	14
1550	De Guzman's	+	1	14
	Anderson's	+	2	14
	Carbon LTD	+	1	14
	S-SSMAVIT	++	60	11
	L-SSMAVIT	+	<1	14
2334	De Guzman's	+	25	14
	Anderson's	+	1	14
	Carbon LTD	+	2	14
	S-SSMAVIT	+++	10	14
	L-SSMAVIT	++	<1	14
2349	De Guzman's	++	50	11
	Anderson's	+	<1	14
	Carbon LTD	+	<5	14

Table 3.15 **Extent of Growth and Sporulation of the NCIB Strains of *B.stearothermophilus* on Different Sporulation Media**

NCIB Strain Number	Sporulation Medium	Growth	Sporulation (%)	Maximum by Day :
	S-SSMAVIT	+++	<5	14
	L-SSMAVIT	++++	10	14
8222	De Guzman's	+++	60	12
	Anderson's	++	20	14
	Carbon LTD	++	40	14
	S-SSMAVIT	+/-	0	14
	L-SSMAVIT	+	0	14
8920	De Guzman's	++	10	14
	Anderson's	+/-	0	14
	Carbon LTD	++	60	6
	S-SSMAVIT	+++	0	14
	L-SSMAVIT	++++	1	14
8922	De Guzman's	++	10	14
	Anderson's	++	1	14
	Carbon LTD	+	<2	14

the pellets combined and resuspended in double distilled water in one tube. The centrifugation process was repeated to form one large pellet. This pellet consisted of 3 layers. The bottom was dark, and contained cell debris plus any intact vegetative cells containing spores. The top layer was a cream colour and consisted of intact , unsporulated vegetative cells. The middle layer was light grey to pink in colour and contained free spores. In a good crop, the middle layer was larger than the other two. The top layer was removed by gently swirling the supernatant to resuspend the lightly packed vegetative cells. This supernatant was then discarded and another 10 ml of cold SDW was added. This water was swirled until approximately 95% of the middle layer was resuspended. This suspension was then decanted into a fresh centrifuge tube. The whole centrifugation process was then repeated once more, and any layers removed as described above. The final spore crop was examined by microscopy to ascertain its cleanliness. When there was less than 1% vegetative cells the concentration was adjusted to approximately 1 x 10^8 spores/ml by the total count method (section 2.3.1.3). The batch was then assigned a batch number, and stored at 4°C in the dark.

3.3 EXPERIMENTAL

3.3.1 Determination of Percentage Sporulation

The density of growth and the percentage of spores in relation to vegetative cells were recorded prior to harvesting, for each spore batch.

3.3.2 Determination of Ease of Cleaning

During the harvesting and cleaning stage (section 3.2.5), some

spore batches formed discrete layers of spores and debris allowing easy separation whilst other batches formed indistinct boundaries or did not pack down well causing difficulties in cleaning. Each spore batch was rated in terms of ease of cleaning as :- Easy (1-3 washes), Fair (4-8 washes), Bad (8+ washes).

3.3.3 Determination of Growth Index

It is a well known characteristic of bacterial spores that not all of the spores that are inoculated onto solid medium will produce a visible colony. Gould *et al* (1968) have shown that many bacterial spore populations have a proportion which are reluctant to germinate, this proportion are often referred to as superdormant (Russell, 1982).

The production of a visible colony from a bacterial spore requires several events to occur. Firstly the spore must be activated, either by physical or chemical stimulation (Gould, 1984), this being termed germination. The spore must then swell and split to allow growth of the vegetative cell, which is termed outgrowth. In other studies (Chinyanganya, 1989; Hoxey, 1984), the proportion of spores which form colonies has been termed the Germination Index (GI).

As it has been shown that spores that germinate do not necessarily outgrow (Keynan, 1973), it would be more reasonable to rename GI as Growth Index (GI) as a practical measurement of the percentage of spores which produce visible colonies. It must also be noted that the GI is only valid if the conditions of its determination are stated as it has been demonstrated (Chinyanganya, 1989) that the GI can vary according to the growth medium used for the determination.

For each spore batch the viable and total counts were determined as described in sections 2.3.1.2 and 2.3.1.3 and the Growth Index

calculated as :

$$GI = \frac{\text{Viable count}}{\text{Total count}} \times \frac{100}{1}$$

A sample set of data for the determination of the GI of *B. stearothermophilus* ATCC 15952 sporulated on Anderson's medium is shown in Table 3.16. The coefficient of variation of 3.75% indicates that this method is reproducible and so is valid for use in this study.

3.3.4 Determination of Resistance of *B. stearothermophilus* Spore Batches To Inactivation by 0.5% w/v Aqueous Formaldehyde at 70°C

3.3.4.1 Preparation and Standardisation of Aqueous Formaldehyde Solutions

Standard solutions were prepared by diluting 1.316 ml of a 38% w/v formalin solution (assayed as described in section 2.3.3) in sterile glass distilled water to a final volume of 100 ml. The standard solution was placed in a waterbath at 70°C to allow full equilibration for 12 h. This time allows the polymers which exist in formalin solution to break down to monomeric formaldehyde.

3.3.4.2 Determination of Formaldehyde Resistance

19.8 ml of the equilibrated formaldehyde solution was pipetted into a 100 ml glass round-bottomed flask, which incorporated two sampling ports. A sterile magnetic flea was placed in the bottom, and the flask was fitted with a condenser unit (Quickfit, Fisons Ltd,) to prevent loss of formaldehyde by evaporation. The vessel and condenser were held in a retort assembly, and the bottom of the vessel was immersed in a water bath at 70°C. A magnetic stirrer

Table 3.16 Quintiplicate Determinations of Growth Index for Spores of *B. stearothermophilus* ATCC 15952 Produced on Anderson's Medium to Demonstrate the Reproducibility of the Determination

Sample	Viable Count (Colonies/ml)	Total Count (Spores/ml)	GI (%)
1	1.83×10^8	3.46×10^8	52.9
2	1.79×10^8	3.58×10^8	50.0
3	1.94×10^8	3.49×10^8	55.6
4	1.86×10^8	3.52×10^8	52.8
5	1.97×10^8	3.72×10^8	53.0
Mean GI (%)			52.86
Standard Deviation			1.98
Coefficient of Variation (%)			3.75

(Rank Bros. Ltd, Cambridge, England) was immersed under the vessel inside the water bath. This was adjusted to turn the magnetic flea at approximately 90 rpm to stir the formaldehyde solution in the vesel. 0.2 ml of the spore suspension was aseptically added through one of the sampling ports. The volume of spore suspension was calculated to give a starting concentration of approximately 1×10^6 spores/ml. A parallel dilution in SDW allowed the determination of the initial viable count at time zero. Throughout the inactivation experiment, 1 ml samples of the inactivation mixture were taken *via* the sampling port at determined time intervals. These samples were aseptically added to 9 ml of the inactivating solution (10% w/v glycine), which had been demonstrated to inactivate the formaldehyde carried over in the sample (Hoxey, 1984). This mixture was vortexed, then left to stand for a minimum of two minutes before appropriate serial dilutions in SDW were carried out. Triplicate aliquots (2 ml) of the final three dilutions were filtered through 47mm diameter, 0.45 micron cellulose nitrate filters (Whatman, Maidstone). The filters were housed in negative pressure filter holders on a vacuum manifold (Sartorius, Epsom). The filters were washed with three 5 ml volumes of SDW to remove any excess glycine which would inhibit recovery of the spores. The membranes were then aseptically transferred, using forceps, onto the surface of overdried Nutrient Agar plates. These plates were then incubated at 55°C for five days. The number of colony forming units (CFU) were then counted, and the percentage survivors relative to the zero-time count calculated. From these results semi-log plot survivor curves were constructed. Survivor curves were constructed by plotting the percentage survivors on a Log scale (Y-axis) against exposure time on a linear scale (X-axis).

To demonstrate the reproducibility of this technique, three exposure experiments were carried out on spores of *B. stearothermophilus* ATCC 12016 sporulated on solid SSMAVIT medium. A survivor plot was constructed and is illustrated in Figure 3.1. In this example, the survivor plot illustrated is non-linear. Nevertheless, the plot illustrated here demonstrates the reproducibility of the experimental method used for determining the resistance of spores to 0.5% aqueous formaldehyde at 70°C. To compare linear survivor curves, it is usual to compare the D-values of each line (the time for a 1 log drop in the surviving fraction). However, in the comparison of non-linear survival curves, the D-value is not a good measure of the resistance of an organism, as it takes no account of shoulders or tails. For this reason, a value called the T_3 value is calculated. This is defined as the exposure time taken for the fraction of survivors to be reduced by three log cycles (99.9%). This should give an average measure of the resistance of an organism, and has been used successfully by Chinyanganya (1989). The T_3 value for the example in Figure 3.1 is 32 minutes.

The method for determining the formaldehyde resistance of spores, described in section 3.3.4.2 was then used to construct survivor curves for all of the "successful" spore batches produced in Section 3.2.5. The T_3 values for all of these plots were then calculated.

Table 3.17 summarises all of the characterisation studies carried out in this chapter, including GI, Ease of Cleaning, T_3 value and type of survivor curve. The individual plots of the survivor curves are illustrated in Appendix II. A representation of each of the major types of curve obtained are shown in Figure 3.2. and are discussed fully in section 3.4. From the characteristics listed in Table 3.17 (a & b), it was possible to choose two possible choices

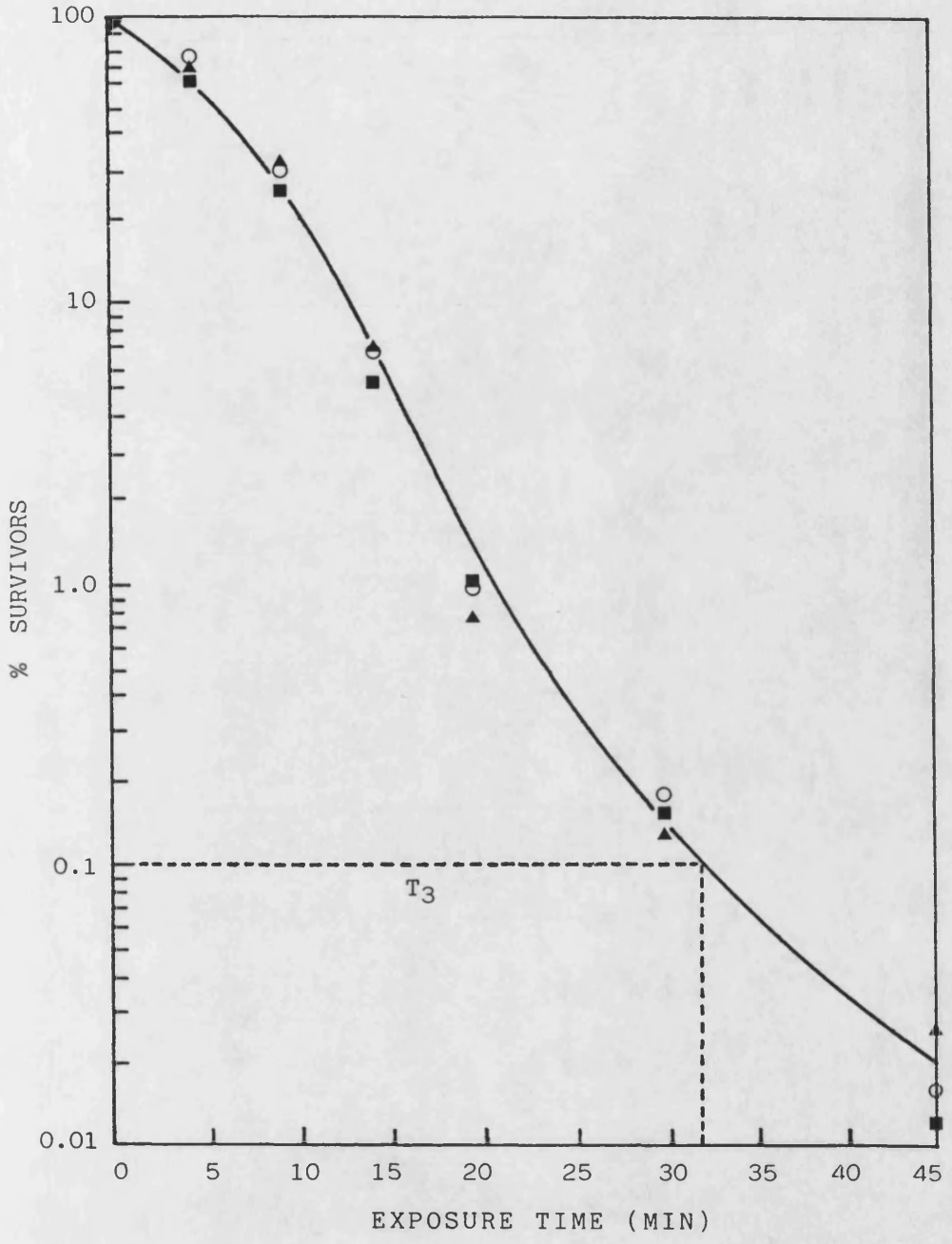


Figure 3.1 Survivor Curve for Three Replicate Inactivation Experiments of Spores of B. stearothermophilus ATCC 12016 Produced on Solid SSMAVIT Medium When Exposed to 0.5% Aqueous Formaldehyde at 70°C.

Table 3.17 (A) Summary Table of All Characteristics of Batches of Spores of ATCC Cultures Produced on Defined Media

Strain No.	Batch No.	Sporulation Medium	Growth	Sporulation		GI* (%)	Ease of Cleaning	T_3* (Min)	Type of Curve*
				%	Days to Max.				
ATCC 12016	BA15	S-SSMAVIT	++++	95	6	40	Easy	34	B
ATCC 12016	BA4	C-LTD	++	90	9	16	Fair	29	D
ATCC 15952	BA2	L-SSMAVIT	++	90	6	63	Bad	29	C
ATCC 15952	BA3	Anderson's	++	90	4	53	Fair	46	E
ATCC 15952	BA14	S-SSMAVIT	++++	90	3	64	Easy	25	D
ATCC 21365	BA5	C-LTD	++	90	6	74	Fair	21	D
ATCC 21365	BA8	De Guzman's	+++	90	6	48	Easy	25	D
ATCC 21365	BA13	S-SSMAVIT	++++	95	3	3	Easy	32	D

Key : S-SSMAVIT - Solid SSMAVIT
L-SSMAVIT - Liquid SSMAVIT
C-LTD - Carbon LTD

++++ = Profuse
+++ = Good
++ = Poor
+ = Poor/none

Easy - 1-3 Washes
Fair - 4-8 Washes
Bad - > 8 Washes

* - Average of three determinations

Table 3.17 (B) Summary Table of All Characteristics of Batches of Spores of NCIB and DSM Cultures Produced on Defined Media

Strain No.	Batch No.	Sporulation Medium	Growth	Sporulation		GI (%)*	Ease of Cleaning	T_3 (Min)*	Type of Curve*
				%	Days to Max.				
NCIB 8222	BA21	De Guzman's	+++	60	10	3	Easy	60	A
NCIB 8222	BA22	C-LTD	++	50	13	1.7	Easy	50	E
NCIB 8224	BA25	C-LTD	++++	80	2-3	65	Easy	111	D
NCIB 8920	BA7	C-LTD	++	60	6	23	Fair	45	D
DSM 458	BA19	S-SSMAVIT	+++	60	11	46	Easy	47	B
DSM 458	BA20	L-SSMAVIT	++	50	13	1.3	Bad	80	B
DSM 2334	BA17	S-SSMAVIT	++	60	11	90	Easy	35	D
DSM 2349	BA18	De Guzman's	++	50	11	90	Easy	20	D/E

KEY :

S-SSMAVIT - Solid SSMAVIT
L-SSMAVIT - Liquid SSMAVIT
C-LTD - Carbon LTD

++++ = Profuse
+++ = Good
++ = Poor
+ = Poor/none

Easy - 1-3 Washes
Fair - 4-8 Washes
Bad - > 8 Washes

* - Average of three determinations

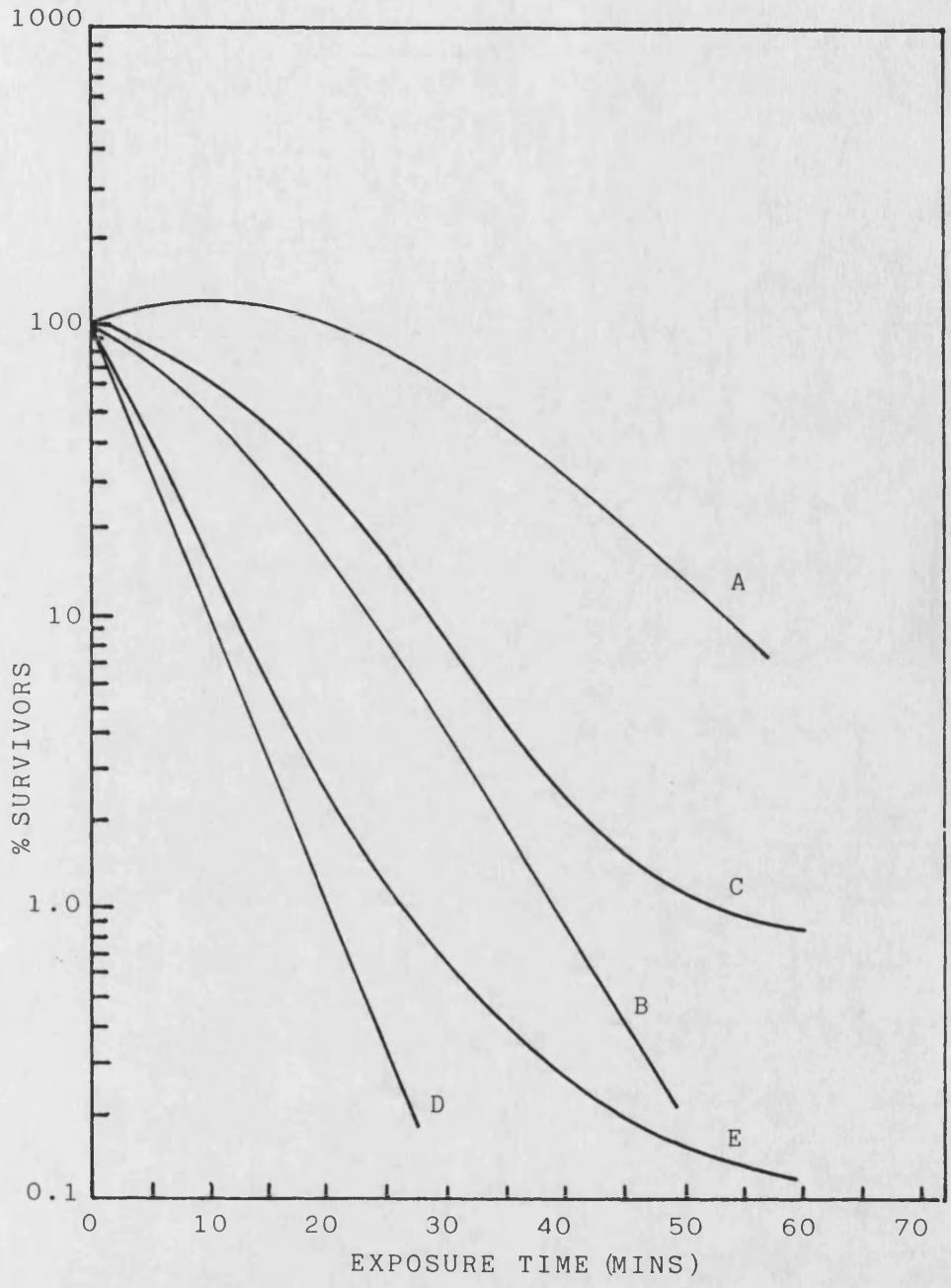


Figure 3.2 Different Types of Survivor Curve Shape Obtained During the Inactivation Studies Using 0.5% w/v Aqueous Formaldehyde

for use in further studies to develop a Biological Monitor for LTSF. The first choice was NCIB 8224 sporulated on Carbon LTD medium and the second being ATCC 15952 sporulated on Solid SSMAVIT. The reasons for the choice of these organisms are discussed in section 3.4.

3.3.5 Reproducibility of Spore Batches

From the data shown previously (Table 3.17 a & b) it was possible to pick out two likely candidates for use in a Biological Monitor. However, if these organisms are to be considered as potential Biological Indicator organisms for LTSF, the reproducibility of the Growth Indices and survivor curves both within and between batches must be demonstrated.

To demonstrate the reproducibility of the Growth Indices, the GI's of three batches of *B. stearothermophilus* NCIB 8224 sporulated on Carbon LTD medium and four batches of ATCC 15952 sporulated on Solid SSMAVIT, were determined. The results of these are recorded in Table 3.18 (A and B). For both of these sets of data, the coefficient of variation was calculated. The values of 4.1% for *B. stearothermophilus* ATCC 15952 and 3.7 % for NCIB 8224 are both below 5%, and this is considered an acceptable degree of variation.

To determine the reproducibility of the survivor curves, a minimum of three replicate survival experiments were carried out on three separate batches of *B.stearothermophilus* NCIB 8224 Sporulated on C-Ltd, and three batches of ATCC 15952 sporulated on solid SSMAVIT.

Table 3.18 Reproducibility of Growth Indices for Different Spore Batches of *B. stearothermophilus*.

a) ATCC 15952 Produced on Solid SSMAVIT Medium

Batch Number	Growth Index (%)
BA23	57
BA27	61
BA28	56
BA29	60
Mean	58.5
Standard Deviation	2.38
Coefficient of Variation	4.1%

b) NCIB 8224 Produced on Carbon Limited Medium

Batch Number	Growth Index (%)
BA24	68
BA25	65
BA26	70
Mean	67.7
Standard Deviation	2.51
Coefficient of Variation	3.7%

3.3.5.1 Treatment of Results

When a linear relationship is assumed to exist between two variables (For example, exposure time and Log % survivors), it is usual to calculate the best fit straight line by regression analysis. The simplest mathamatical model for this assumes that the independant variable (Exposure time) is known without error of measurement, and that the corresponding measured values of the dependant variable (Log % survivors) are scattered normally from their true values. The method of calculating a least squares regression analysis for this type of data is described in Appendix III.

As the data from the experiments described in section 3.3.5 exhibited a linear relationship, they were analysed by least squares regression analysis. The intercepts, slopes and correlation coefficients obtained from these analyses are shown in Table 3.19 and Table 3.20. The correlation coefficient is a measure of how well the data fit a linear model. All of those obtained here were greater than 90% fit, and therefore the linear model can be accepted as a good one.

To demonstrate that none of the individual survivor plots were significantly different it was necessary to compare the slopes and intercepts. This was done using analysis of variance. As the data fit into a symmetrical 3 (replicates) x 3 (batches) form, a two-way analysis of variance (2 way ANOVA) was used. The method of ANOVA is described in Appendix III.

The results of these analyses are given in Tables 3.21 to 3.24. To test the significance of difference within (replicates on one batch) and between (between batches), the F-value for each must be found in a statistical data table. None of F-values recorded in

Tables 3.21 to 3.24 are greater than those for P=0.05 (5%) for the relevant degrees of freedom. This means that the differences of slope and intercept between batches are no higher than within batches and that neither are significant at a probability level of 5%. Therefore this demonstrates the reproducibility of these survivor curves.

Survivor plots of data for spores of *B.stearothermophilus* NCIB 8224 sporulated on Carbon LTD and ATCC 15952 sporulated on Solid SSMAVIT are illustrated in Figure 3.3 and 3.4. For clarity only the mean points of the nine survivor curves for each organism are shown. Error bars (calculated as described in Appendix III) are also shown to indicate the scatter obtained.

Table 3.19 Intercepts, Slopes and Correlation Coefficients (R^2) Obtained by Regression Analysis, for Survivor Curves of *B. stearothermophilus* ATCC 15952 sporulated on Solid SSMAVIT Medium When Exposed to 0.5% Aq. Formaldehyde at 70°C

Batch	R^2 (%)	Intercept on Y-axis	Slope
BA27	98.1	1.8037	-7.7156×10^{-2}
	96.8	1.8648	-7.1940×10^{-2}
	99.8	1.8998	-6.7000×10^{-2}
BA28	98.6	1.9010	-6.9737×10^{-2}
	98.4	1.8584	-7.1563×10^{-2}
	95.8	1.7384	-6.7896×10^{-2}
BA29	98.7	1.9368	-8.3000×10^{-2}
	97.6	1.8628	-6.5627×10^{-2}
	99.3	1.8615	-7.7556×10^{-2}

Table 3.20 Intercepts, Slopes and Correlation Coefficients (R^2) Obtained by Regression Analysis, for Survivor Curves of *B. stearothermophilus* NCIB 8224 sporulated on Carbon LTD Medium When Exposed to 0.5% Aq. Formaldehyde at 70°C

Batch	R^2 (%)	Intercept on Y-axis (Log % survivors)	Slope
	97.7	2.0438	-2.6368×10^{-2}
BA25	98.9	1.8961	-2.3842×10^{-2}
	98.6	1.9853	-2.8850×10^{-2}
	97.2	1.8665	-2.6471×10^{-2}
BA26	99.3	1.8857	-2.4476×10^{-2}
	98.7	1.9843	-2.5823×10^{-2}
	99.9	1.9912	-2.2059×10^{-2}
BA24	98.9	1.9571	-2.2260×10^{-2}
	95.4	2.0488	-2.8741×10^{-2}

3.4 Discussion

The results for growth and sporulation of the different strains of *B. stearothermophilus* on the different chemically defined media are given in Tables 3.1-3.3. The extent of growth and sporulation varied greatly, both from strain to strain and on different media.

The composition of all of these media is based upon the knowledge that specific nutrients are required for growth and sporulation. It

Table 3.21 Two-way Analysis of Variance of slopes of Survivor Curves for Spores of Three Batches of *B. stearothermophilus* ATCC 15952 sporulated on Solid SSMAVIT medium

Batch	Slopes		
1	-7.7156×10^{-2}	-7.1940×10^{-2}	-6.7000×10^{-2}
2	-6.9737×10^{-2}	-7.1563×10^{-2}	-6.7896×10^{-2}
3	-8.3000×10^{-2}	-6.5627×10^{-2}	-7.7556×10^{-2}

Analysis of Variance

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Between Samples	2	8.2925×10^{-5}	4.1463×10^{-5}	1.2
Within Samples	2	4.8657×10^{-5}	2.4329×10^{-5}	0.7
Residual	4	1.3330×10^{-4}		
Total	8	2.64885×10^{-4}		

For between : $F(2, 4)\ P_{0.05} = 6.59$

For within : $F(4, 2)\ P_{0.05} = 19.25$

Table 3.22 Two-way Analysis of Variance of Intercepts of Survivor Curves for Spores of Three Batches of *B. stearothermophilus* ATCC 15952 sporulated on Solid SSMAVIT medium

Sample	Intercepts (Log % survivors)		
1	1.9010	1.8584	1.7384
2	1.8037	1.8648	1.8998
3	1.9368	1.8628	1.8615

Analysis of Variance

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Between Samples	2	3.4039×10^{-3}	1.7020×10^{-3}	0.4
Within Samples	2	4.4721×10^{-3}	2.2361×10^{-3}	0.5
Residual	4	1.9261×10^{-2}	4.8152×10^{-3}	
Total	8	2.7137×10^{-2}		

Coefficient of Variation = 3.7%

$F(4, 2)\ P_{0.05} = 19.25$

Table 3.23 Two-way Analysis of Variance of Slopes of Survivor Curves of Three Batches of Spores of *B. stearothermophilus* NCIB 8224 Sporulated on Carbon LTD medium

Batch No	Slope of Survivor Curve		
1	-2.6300×10^{-2}	-2.3842×10^{-2}	-2.8850×10^{-2}
2	-2.6471×10^{-2}	-2.4476×10^{-2}	-2.5823×10^{-2}
3	-2.2059×10^{-2}	-2.2260×10^{-2}	-2.8741×10^{-2}

Analysis of Variance

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Between Samples	2	2.8439×10^{-5}	1.4219×10^{-5}	3.8
Within Samples	2	6.1120×10^{-6}	3.0560×10^{-6}	0.8
Residual	4	1.5071×10^{-5}	3.7677×10^{-6}	
Total	8	4.96215×10^{-5}		

$F(4, 2)\ P_{0.05} = 19.25$

Table 3.24 Two-way Analysis of Variance on the Intercepts of Survivor Curves of three batches of spores *B. Stearothermophilus* NCIB 8224 Sporulated on Carbon LTD

Batch No	Intecepts (Log % survivors)		
1	1.9912	1.9571	2.0488
2	1.8657	1.8865	1.9843
3	2.0438	1.8960	1.9853

Analysis of Variance

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Between Samples	2	1.3062 x 10-2	6.531 x 10-3	2.3
Within Samples	2	1.2079 x 10-2	6.039 x 10-3	2.5
Residual	4	1.0335 x 10-2	2.584 x 10-3	
Total	8	3.5477 x 10-2		

Coefficient of Variation = 2.6%

$F(2, 4)\ P_{0.05} = 6.94$

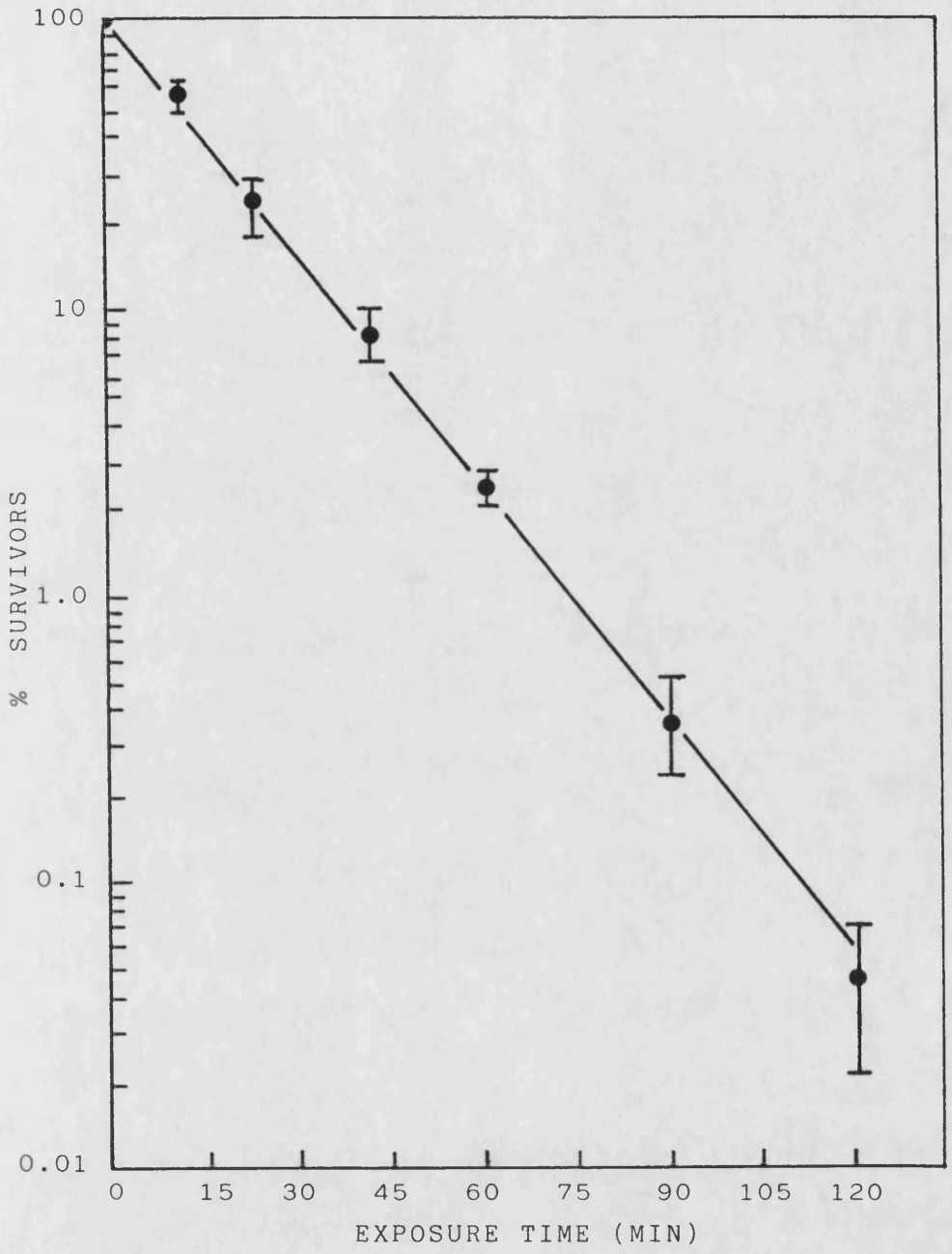


Figure 3.3 Mean Survivor Curve for Spores of B. stearothermophilus NCIB 8224, Produced on C-limited Medium, When Exposed to 0.5% Aqueous Formaldehyde at 70°C

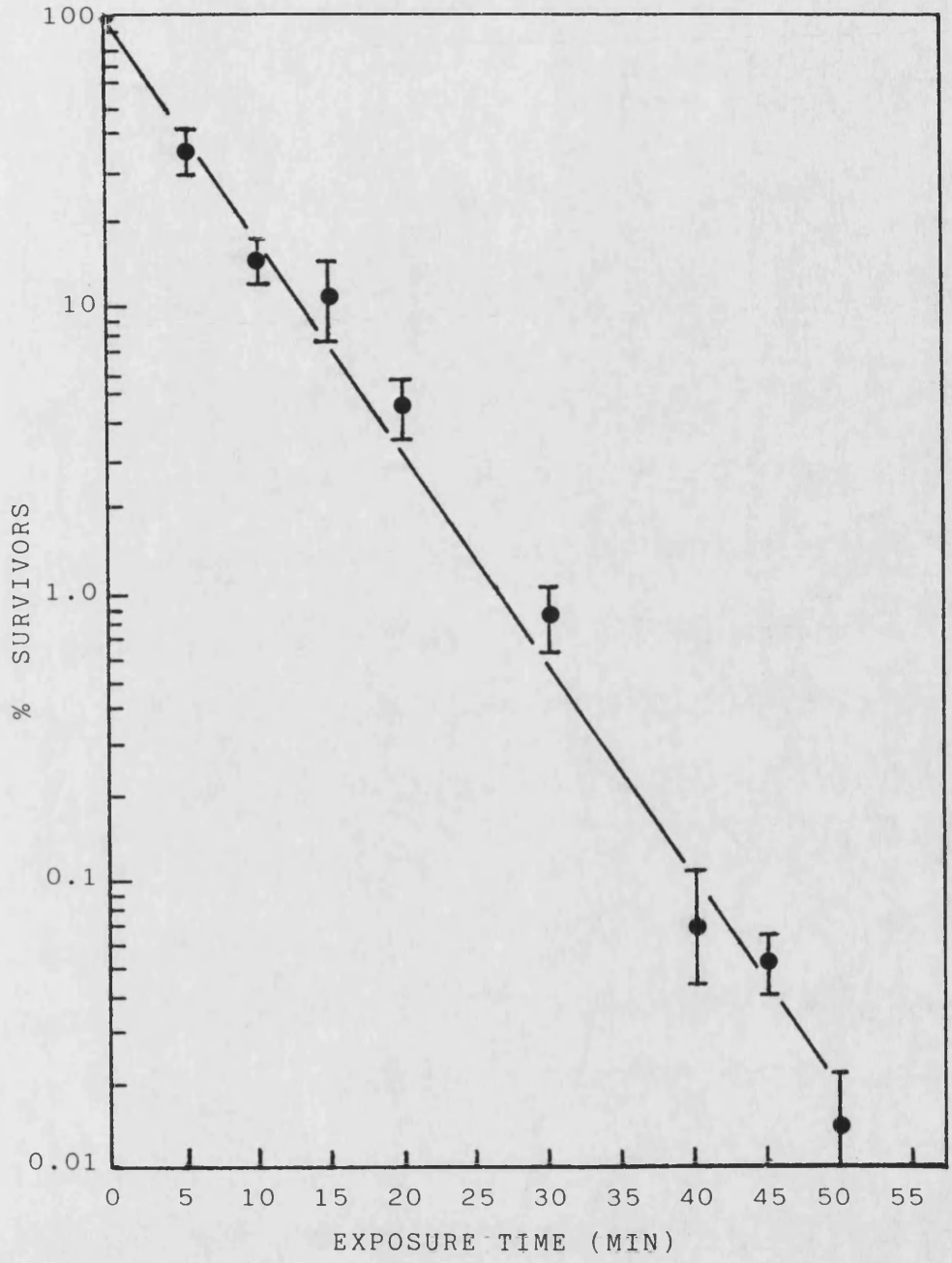


Figure 3.4 Mean Survivor Curve for Spores of B. stearothermophilus ATCC 15952, Produced on Solid SSMAVIT Medium, When Exposed to 0.5% Aqueous Formaldehyde at 70°C

has been reported that a wide range of amino acids are required to support growth of thermophilic organisms (Campbell and Williams, 1953b; O'Brien and Campbell, 1957; Baker *et al*, 1960). It has also been reported that specific minerals and amino acids are required to produce spores (Anderson and Friesen, 1972). More specifically much research has been devoted to finding out which specific nutrients, when limited, initiate sporulation. One such piece of work stated that limitation of metabolizable carbon, nitrogen, phosphate or sulphate would initiate sporulation in *Bacillus* spp. (Lee and Brown, 1975). Other work has shown the requirement of specific minerals for certain spore characteristics, for example calcium affects spore heat resistance (Murrell, 1969), as does iron, magnesium and phosphate (Murrell and Warth, 1965).

In the results obtained in this study, Anderson's medium was found to be the worst medium in terms of volume of growth and percentage sporulation. This is almost certainly due to the very minimal nature of this medium. The volume of spores produced varied little from about the third day in most cases. At first it would appear that perhaps all the glucose was rapidly used up by the initial inoculum, initiating sporulation before good growth occurred. This appears not to be the case however, as C-limited medium has the same amount of glucose available, and was more successful than Anderson's. These two media are very similar, except that C-limited has more vitamins than does Anderson's. Also Anderson's media is a liquid whereas C-limited is a solid. Ordal (1961) and Halvorson (1962) reported an increasing demand for oxygen as vegetative cells started to sporulate. It has been demonstrated that oxygen is only half as soluble at 55°C as at 30°C (Allen, 1953), and a liquid culture does not expose as much surface area for oxygen exchange as does a solid

medium. Therefore it is probably a combination of low nutrients, few vitamins and little oxygen which contribute to the failure of Anderson's medium.

The best medium in terms of the range of organisms exhibiting both good growth and high percentage sporulation was solid SSMAVIT. This is probably to be expected as this medium was specifically designed to sporulate as many *Bacilli* as possible (Hoxey, 1984). The few exceptions in this study were organisms which exhibited an all round lack of growth and sporulation (e.g. DSM 1550). The drawback of this medium was its complexity, which is understandable considering the aim of its design. An interesting observation is the difference in success of the solid and liquid forms of SSMAVIT. Both have the same chemical composition, but even with the same organism differing results were sometimes obtained (e.g. ATCC 15952). In the case of ATCC 15952, the amount of growth observed on solid media was a lot better than with the liquid form, although the percentage sporulation and number of days to maximum sporulation were the same. This can only be explained by either the lower availability of oxygen in liquid media or perhaps a preference for solid support whilst sporulating. This difference in growth holds for most of the strains tested.

De Guzman's medium was a mixture of success and failure. It was one of the best media for supporting growth, often supporting it even better than SSMAVIT. Unfortunately, however, it has a low success rate at sporulating the strains, often supporting profuse growth for 14 days with little sign of sporulation. The reason for the profuse growth is probably similar as for SSMAVIT. Both are very complex media and both have high glucose concentrations (2.0 g/l). This will support a great deal of growth before the glucose becomes limiting

and initiates sporulation. The lack of sporulation may be due to DeGuzman's medium being the only chemically defined media not containing L-glutamic acid of at least twice the concentration of glucose. Glutamic acid has been shown to be important in stimulating sporulation (Murrell, 1969), and is needed in a relatively high concentration compared to glucose, although only 20% may be used (Anderson and Friesen, 1972)

Table 3.17 (a and b) lists all the "successful" batches produced, and their characteristics such as percentage sporulation, time to maximum sporulation, Growth Index and a summary of the T_3 and types of curves obtained from the graphs.

Shull *et al*, 1963 stated that most organisms obey first order kinetics. This would mean that the majority of survival curves should exhibit log-linear inactivation. This appears to be true in the case of the batches of organisms inactivated by formaldehyde in this study. Though this was the case, there were exceptions, and in all, five different types of survivor curve were exhibited. Typical forms of these types are depicted in Figure 3.2 with the original data in Appendix II and a summary in Table 3.17 (a & b).

Cerf (1977) categorised shapes of survivor curves into six types, illustrated in Figure 1.2. The most common of these types is the log linear or "D" type curve. This is an agreement with Shull *et al*, 1963 in that these types of curve should predominate. These were the most desirable shape for use as a Biological Indicator organism, allowing easy prediction of the amount of "kill" a particular treatment causes. This was exhibited in eight out of sixteen (50%) "successful" combinations of media and organism.

The next most common type of curve was the "shouldered" or type "B" curve. This was found in three out of 16 (<25%) "successful"

combinations. This type of curve is found quite regularly, and is generally described as a result of a combination of some activation mechanism with inactivation (Halvorson, 1958). This could be true, in that all of the batches exhibiting this type of curve have Growth Indices less than 46%, and in particular DSM 458 *ex* liquid SSMAVIT had a G.I. of only 1.3%. This organism therefore had an enormous potential for activation.

The third type of curve exhibited in this study is the "upward concavity" or "E" type curve. A consensus on whether or not this type of curve is an artefact has not been reached. Two theories have been presented, the vitalistic and the mechanistic. The vitalistic theory relies upon the fact that individuals in a population are not identical (Lee and Gilbert, 1918). This would mean a characteristic, such as resistance to a lethal agent, could be possessed to different degrees by different individuals in the population. Another important point in this theory is that the degree of difference in a population is permanent. This means that sub-culturing of resistant survivors would produce a population with the same range of resistance. The "mechanistic theory" assumes that there is a basic similarity of resistances in a population. It supposes that only a proportion of the molecules of the sterilant/disinfectant and the microorganisms are in a state to interact at any one time. Hence, the destruction or inactivation process may be regarded as a series of unimolecular events. This experimental result would be expected to be logarithmic. Generally, the mechanistic explanation is accepted (though this itself has many sub-theories). Whatever is true, other examples of spores which exhibit these characteristic curves such as the spores devoid of exchangeable calcium described by Alderton *et al*, 1964 or various *Bacillus* species produced when

inactivated by moist heat at 110°C (Hoxey, 1984) have been described.

The last two types of curve were only observed in one case each. The first of these was the "shoulder" or "A" type curve. This has the same explanation as the "B" type shouldered curve, i.e. a mixture of activation and inactivation. In the case of an "A" type curve however, the activation mechanism outpaces the inactivation mechanism for a short while. This leads to a percentage of survivors greater than 100% occurring for a short while. After this time, activation slows down, presumably as the number of spores susceptable to activation gets less. The inactivation mechanism then takes over, leading in a normal logarithmic decline thereafter. These types of curves have been reported before (Etoa & Michiels, 1988).

The second of these types is a "sigmoidal" or "C" type curve. This type has both an initial lag phase, and a tail, with a linear portion in between. This was exhibited by ATCC 15952 *ex* liquid SSMAVIT. This appears to be a combination of the "B" and "E" types, with the explanation of both types equally applicable.

In all of these results no correlation can be made between the types of curves obtained and the media used to produce it. It is therefore not possible to predict the shape of curves obtained in inactivation experiments by the media and incubation conditions alone. There also appears to be no correlation between the majority of strains and the type of inactivation curve obtained, or if there is (e.g. ATCC 21365), other characteristics vary widely (Growth Index 3%-74%). It is possible however to substantiate the fact that the sporulation media can alter the characteristics (Lee and Brown, 1975) and resistance (Waites and Bayliss, 1980; Dadd *et al*, 1983a) of the spores. For example, ATCC 15952 sporulated on solid SSMAVIT has a type "D" curve a T_3 value of 25 minutes. For comparison, ATCC 15952

sporulated on Anderson's medium has an "E" type curve with a T_3 value of 46 minutes. Moreover, ATCC 15952 sporulated in liquid SSMAVIT has a "C" type curve with a T_3 value of 28.5 minutes. This could be caused by different media altering the structural components of the spore e.g lipid composition (Long and Williams, 1960; Bayliss *et al*, 1981). This shows that not only media composition, but also the form in which it is presented (liquid or solid) can also greatly influence the characteristics of the spores produced.

The aim of the work presented in this chapter was to choose a suitable candidate for further development into a biological indicator organism for LTSF. The best candidates would have linear inactivation kinetics combined with good resistance to formaldehyde and heat as well as good Growth Index (>60%) and ease of production. Taking these as the criteria, two organisms display the desired characteristics. *B. stearothermophilus* ATCC 15952 sporulated on solid SSMAVIT had a high GI, high yield in a short time (6 days) combined with linear inactivation kinetics and a T_3 value of 25 minutes. Though this T_3 value is not the highest found, it is one of the best combined with a high GI. In fact, GI and T_3 values seem to have an inverse relationship in the results presented here. The use of ATCC 15952 has one drawback, and that is that the SSMAVIT medium is very complex, and therefore would be economically unfavourable for production of a commercial biological indicator. The best of the organisms screened in this study was NCIB 8224 sporulated on C-limited medium. This organism gave a good harvest with high percentage sporulation, was easy to clean and had a T_3 value of 111 minutes. The greatest advantage of this organism, beside its high T_3 value, was that it was produced on a very simple medium. For either of these organisms to be considered as a LTSF monitor, their batch to

batch reproducibility had to be demonstrated. Nine survival experiments were carried out on three batches of spores of each of the two organisms. Tables 3.19 and 3.20 list the slopes and intercepts, calculated by linear regression, of the survivor curves from these experiments. The correlation coefficients, also listed in these tables, are all greater than 95%, which indicates that all the data fitted well to a linear model, hence demonstrating the validity of this analytical method. To demonstrate that there was no significant variation between slopes and intercepts from both the same and different batches of an organism, a two-way ANOVA was carried out on both the slopes and intercepts. The results of these are recorded in Tables 3.21 to 3.24. The F-values obtained from the ANOVA demonstrate that for the relevant degrees of freedom, there is no significant difference between either the slopes or intercepts, both within and between batches of the same organism.

From these results it would appear that the best organism, based on the criteria mentioned earlier, is *B. stearothermophilus* NCIB 8224 sporulated on C-limited medium. *B. stearothermophilus* ATCC 15952 sporulated on SSMAVIT medium is also a reasonably good organism except for the complexity of the medium. For this reason NCIB 8224 was used in all further studies, and ATCC 15952 was kept as a backup organism in case of a failing of NCIB 8224 coming to light further through the investigations.

CHAPTER 4

DEVELOPMENT OF AN EXPERIMENTAL APPARATUS FOR EXPOSURE OF SPORES TO DEFINED LTSF CONDITIONS

4.1. INTRODUCTION

In chapter 3, *Bacillus stearothermophilus* NCIB 8224 sporulated on C-limited medium, was chosen as a potential biological indicator organism for LTSF sterilization, partly on the basis of its linear inactivation kinetics when exposed to aqueous formaldehyde. However, to test its suitability for monitoring the success or failure of an LTSF cycle, it would be necessary to demonstrate that it also exhibited linear inactivation kinetics when exposed to LTSF conditions. This required a test apparatus which would allow samples of the spores to be exposed to defined LTSF conditions, and, to be withdrawn at any time during an LTSF cycle so that the spores could be plated onto recovery media and a survivor curve constructed.

This investigation forms part of a multidisciplinary research project into Low Temperature Steam (LTS) and Low Temperature Steam and Formaldehyde (LTSF) sterilization, these studies being based at Luton College, the PHLS at Norwich and the University of Bath. For this reason it was considered necessary to choose a standard piece of apparatus that could be installed in each laboratory and could run both LTS and LTSF cycles. This would reduce inter-laboratory variation in the data. It would also facilitate the interdisciplinary design of modifications to the apparatus, to improve its working characteristics.

An apparatus that fulfilled these requirements was described previously by Hoxey (1984). The drawback of this equipment was that it required a great deal of skill and maintenance to use, and suffered from condensation problems in the sample vessels. Furthermore, the apparatus was based on a manifold design with multiple sample vessels, and would not be expected to simulate the

conditions that were likely to exist in commercial single chamber LTSF sterilizers.

The apparatus chosen for this investigation was the Miniclave 80 manufactured by Thackeray Ltd, Leeds. It was chosen as it was readily available, cheap, and being a commercial sterilizer it allowed the spores to be tested under conditions to be found in commercially produced sterilizers. Deverill and Cripps (1981) also reported on a test apparatus consisting of a modified Thackray Miniclave 80, though the modifications were not as extensive as those described here.

4.1.1 Operation of Unmodified Miniclave 80

The basic cycle of the Miniclave 80 consisted of several phases. Firstly a vacuum is pulled. This is followed by five steam/vacuum pulses to remove air from the chamber, then approximately 3 ml of formalin is injected followed by a steam hold period during which sterilization takes place. After 20 minutes holding, there is a five pulse steam/vacuum elution followed by a vacuum drying stage. This cycle is illustrated in Figure 4.1. A pipework diagram of the original Miniclave 80 is illustrated in Figure 4.2 (Thackeray Operating Manual 1978), to which reference will be made in the following text.

Switching on the Miniclave 80 energizes the steam/formalin vapouriser (19) to 140°C and the chamber jacket (1) to 75°C, 2°C above the factory set operating temperature of 73°C. The chamber jacket has an over-temperature cut out (16) which is set to 80°C. Once the vapouriser reaches the minimum operating temperature of 120°C, and the door is closed, the "available" and "unsterile" indicator lights become illuminated. The Low Temperature Steam and

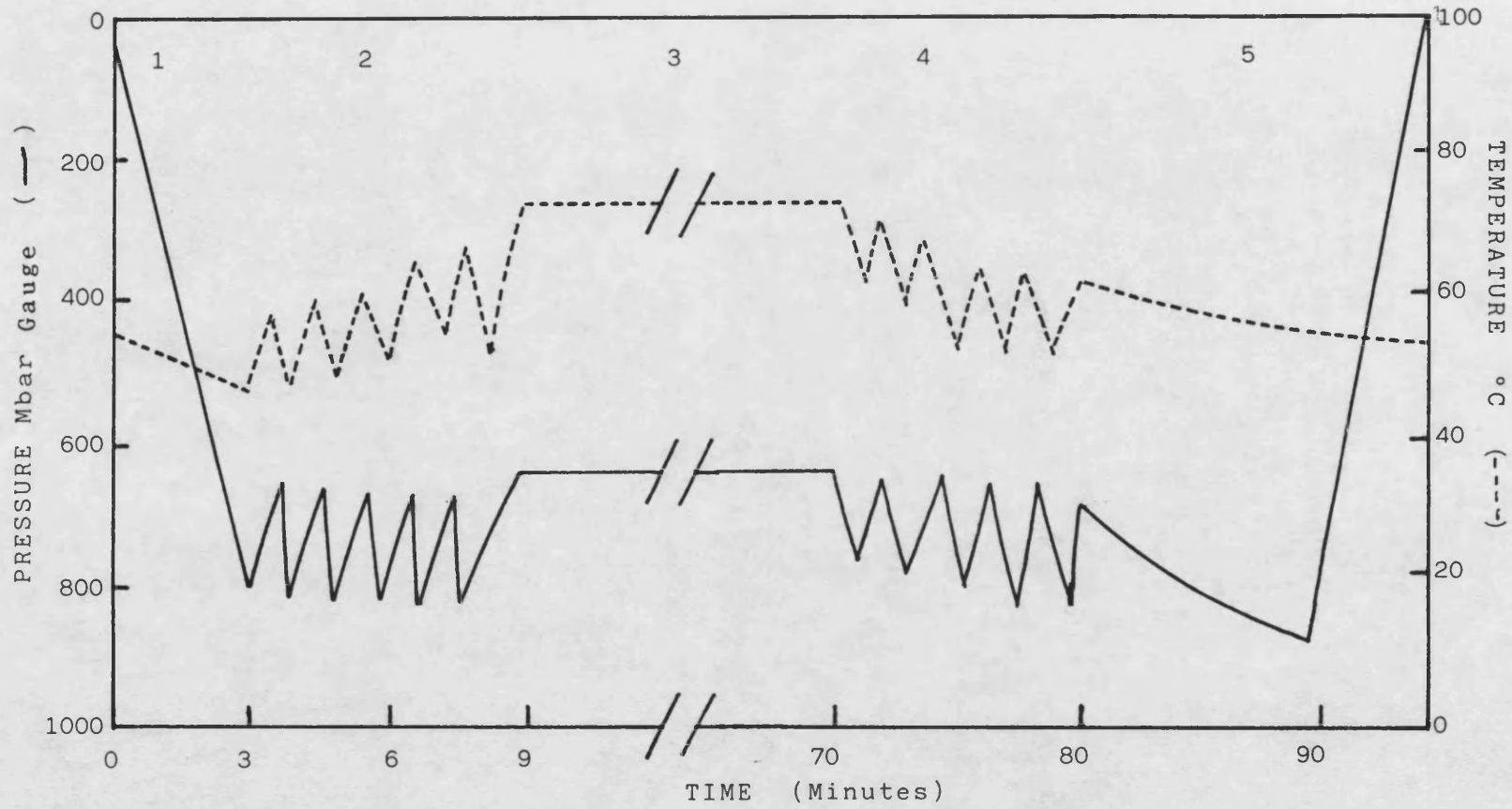


Figure 4.1 Operating Cycle of the Modified Miniclave 80.

Key : 1. Initial Vacuum 2. Steam/vacuum pulses 3. Holding/sterilizing period
4. Elution 5. Drying stage

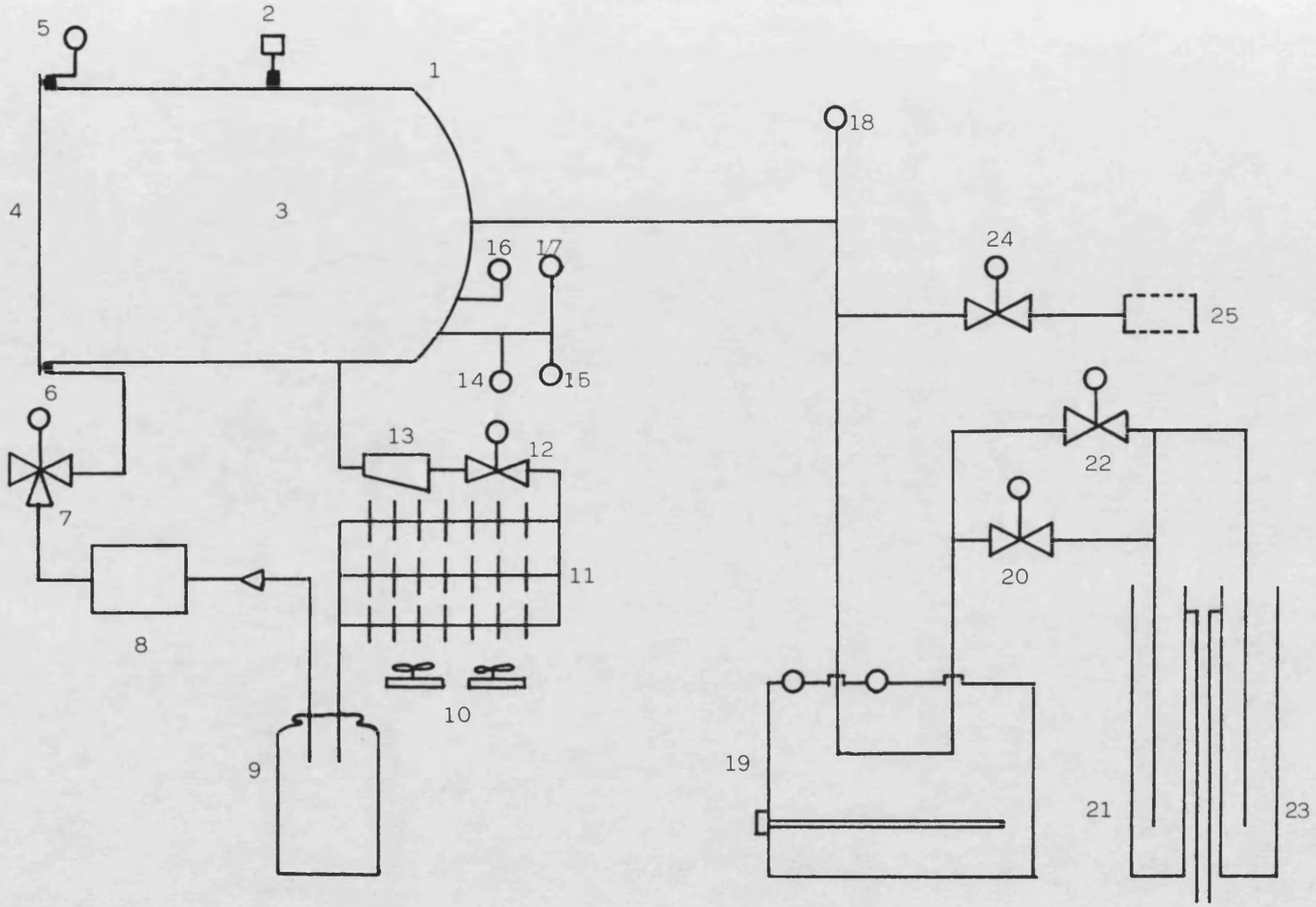


Figure 4.2 Pipework Diagram of the Miniclave 80 as Originally Manufactured. (developed from a diagram in the Miniclave 80 Operating Manual, Thackray)

Key to Figure 4.2 Pipework diagram of the Miniclave 80 as Originally Manufactured.

1. Chamber heating jacket
2. Thermocouple entry port
3. Miniclave 80 chamber
4. Miniclave 80 door
5. Door seal pressure switch
6. Doorpressure `o` ring seal
7. Three way exhaust valve
8. Vacuum pump
9. Vacuum trap bottle
10. Cooling fans
11. Heat exchanger
12. Drain solenoid valve
13. Strainer
14. Vacuum switches
15. Vacuum gauge
16. Temperature thermostat and cut out switch
17. Temperature gauge
18. Vacuum control switch
19. Water/formalin vaporiser
20. Formalin entry solenoid valve
21. Formalin reservoir
22. Water entry solenoid valve
23. Water reservoir
24. Air inlet solenoid valve
25. Air filter

Formaldehyde cycle is selected by the steam/formalin rocker switch. Once the "start" button is pressed, the positive back pressure from the vacuum pump (8) is directed to the door "O" ring (6), pressurising it to 25 lb/in^2 detected by the pressure switch (5), to form a seal against the door, at which point a vacuum is drawn in the chamber (3). At this point the "available" light goes out and the "air removal" light is illuminated. The chamber is now subjected to five steam/vacuum pulses between the pressures of 620 and 500 mm Hg. The vacuum is controlled by a pressure switch (18) controlling a solenoid valve (12) and the steam is supplied by a solenoid valve (22) opening to allow water from the reservoir (23) into the vapouriser and from there to the chamber. Once the pulses have finished, the "steam to chamber" indicator is illuminated, and a valve (20) controlled by timer T2 is opened for two seconds to allow approximately 3 ml of 38% formalin from the reservoir (21) to be drawn by the vacuum into the vapouriser. Steam is now allowed into the chamber until 73°C is achieved, at which point the "temperature achieved" indicator light is illuminated. The holding timer T1 is now energized and very small steam/vacuum pulses are used to maintain the chamber temperature at 73°C +/- 2°C until T1 has elapsed (20 min). The "elution" indicator light is now illuminated and a series of five steam/vacuum pulses, identical to the air removal ones described previously, occur. On completion of elution, the "drying" indicator light is illuminated and the chamber is subjected to a vacuum of 620 mm Hg for 8 minutes controlled by timer T3. This removes any steam and condensate out of the chamber to a condenser (11) and from there to a collection bottle (9). This protects the vacuum pump from condensation. Once timer T3 has elapsed the cycle completes by opening a solenoid valve (24), allowing air into the

chamber through an air filter (25), at which point the door may then be opened and the load removed.

4.1.2 Apparatus Requirements

The Miniclave 80 cycle as described above did not meet the requirements needed for it to be used as an experimental test apparatus. These requirements are:-

1) the ability to introduce and remove samples into the chamber atmosphere without interrupting the cycle,

2) variable temperature control of both jacket and chamber to within +/- 1°C,

3) variable and accurate measurement of formaldehyde volume injected,

4) the ability to sample chamber atmosphere to ascertain conditions within, during the cycle,

5) the ability to measure the chamber temperature at the point at which the samples were held.

To enable the Miniclave 80 to meet these requirements, various modifications were necessary.

4.2 MATERIALS AND METHODS

4.2.1 Modification of the Door

To allow the introduction and removal of samples to the chamber atmosphere five ports, each fitted with a 3/4 inch ball valve, were inserted into the door plate (illustrated in Figure 4.3). To each port was fitted a short length of 1/4" o.d. copper pipe connected to a T piece. To one arm of the T piece was attached a 1/4" ball valve fitted with a Millipore filter (Swinnex filter with ap 2501300 paper prefilter) open to the atmosphere. The other arm of the T piece was

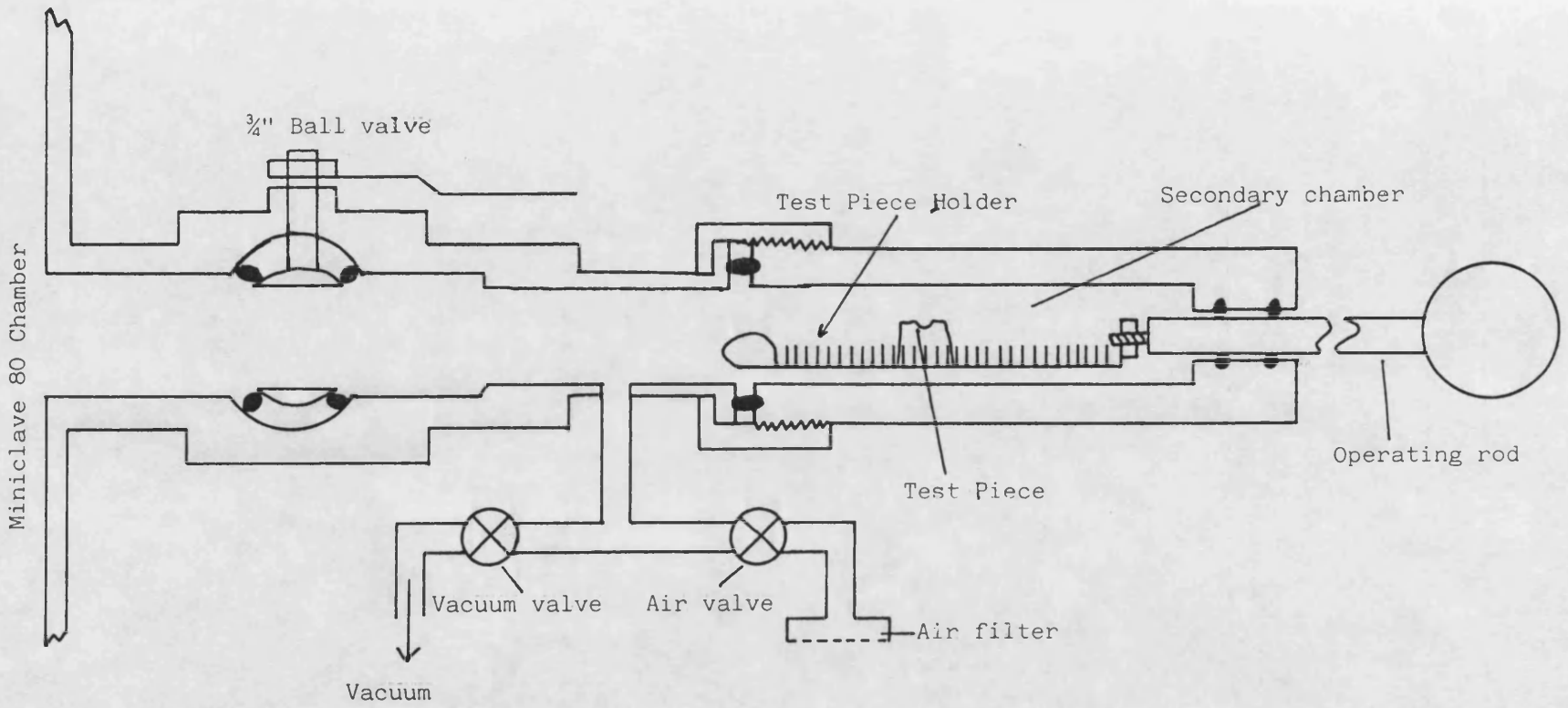


Figure 4.3 Chamber Atmosphere Sampling System (after Line, 1989)

connected via a 1/4" ball valve to a vacuum line from the Miniclave 80 vacuum pump.

A secondary chamber, illustrated in Figure 4.3, was constructed out of brass to allow it to screw onto the 3/4" ball valve of the port. A 7 mm diameter steel rod was fitted to slide axially through the secondary chamber, and when the door port 3/4" valve was open it could slide into the Miniclave 80 chamber. Attached to the chamber end of the rod was the test piece holder. This consisted of a spring of approximately 4cm length supported by a wire backbone (illustrated in Figure 4.3). This allowed the "legs" of the test pieces prepared in 4.3.2 to be held firmly in the spring. The angle of the ports fitted in the door plate were such that the test piece holders inserted into the Miniclave 80 chamber, through each of the door ports, would meet in the centre of the chamber. This should minimise differences in exposure conditions of test pieces in the chamber. A photograph of the Miniclave door with all the ports attached is shown in Plate 4.1.

4.2.2 Modification of the Steam Supply and Chamber Temperature Control

The steam supply to the Miniclave 80 chamber was originally from the steam/formalin vaporiser. This was suspected of not supplying good quality dry saturated steam. Also, as the vaporiser was supplied from a small volume water reservoir it could not run more than one cycle without being topped up with water. To overcome this problem the water reservoir was removed and the steam inlet pipe to the chamber was connected *via* a 1/4" ball valve to a "Little Sister" autoclave (Thackray, Leeds) which has been modified to supply a constant head of steam at 110°C. As a finite time was required for

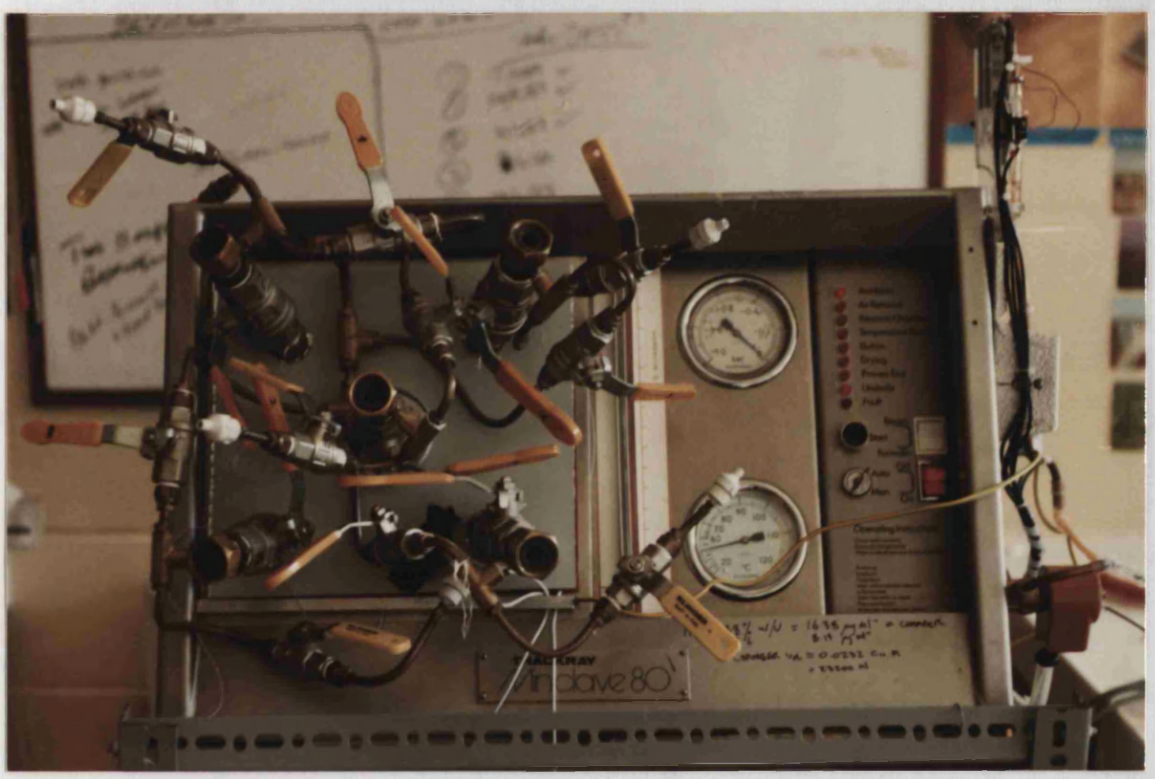


Plate 4.1 Photograph of the front of the Miniclave 80 Illustrating the Arrangement of the Pipework and Sampling Ports on the Chamber Door

the thermocouple and temperature detecting apparatus in the Miniclave 80 to react to temperature changes, a ball valve was used to restrict the flow of steam into the chamber thereby giving a more gradual increase in temperature and hence minimising overshoot of the set temperature.

The steam is allowed into the chamber *via* a solenoid valve (24). The original controller of this valve, a pressure switch, was replaced with a Cal 9000 (RS Components Ltd) temperature thermostat. This sensed the chamber temperature using a copper/constantan thermocouple located in the drain of the chamber. The drain is considered the coolest part of the chamber, assuring that the minimum temperature in the Miniclave 80 chamber is that at which the sterilizing process is to be run. The range of cycle temperature and pressure these modifications allowed is illustrated in Figure 4.4.

4.2.3 Modification of the Jacket Temperature Control

The original side mounted jacket temperature controller was replaced with a calibrated proportional temperature controller (RS Components Ltd), which sensed the temperature of the chamber wall inside the jacket on the outside of the chamber. This was calibrated against a digital thermometer (Bibby Ltd.) on the inside of the chamber wall to ensure that the inner wall attained the temperature set on the controller.

4.2.4 Addition of a Door Heater and Controller

Even with the modifications described above, there was found to still be a problem with condensation occurring within the chamber. The main cause of the condensation was the chamber door and its

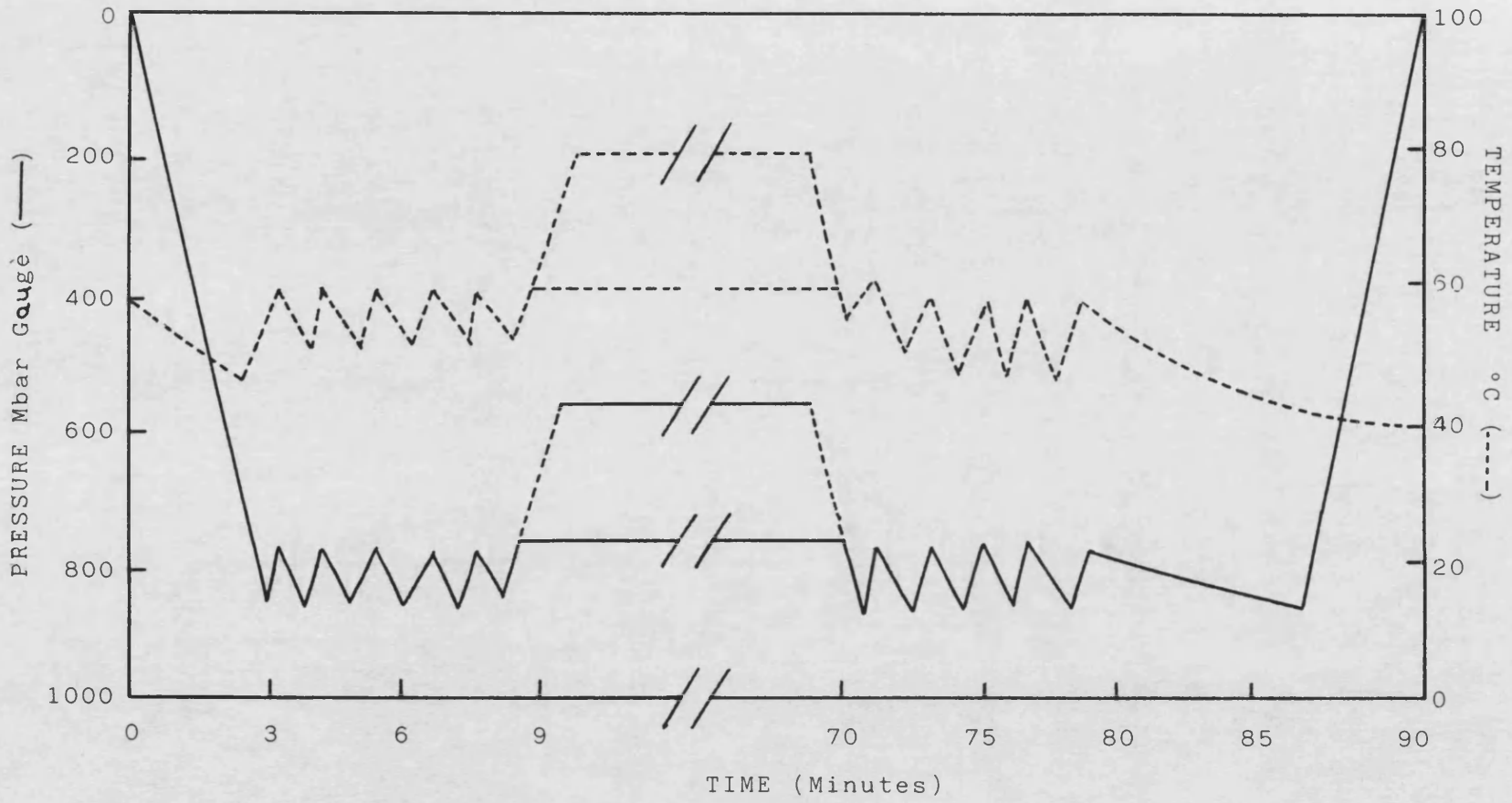


Figure 4.4 Range of Operating Temperatures, Pressures and Holding Times Possible in the Modified Miniclave 80.

fixtures (ports, etc.), which provided a large heat sink. Handlos (1979) stated that all the surfaces of the chamber must be heated to prevent condensation. It therefore was considered necessary to heat the door to the same temperature as the jacket, and for this purpose a tailor made rubber mat containing heating elements (Holroyd components Ltd, Saffron Walden, England) was glued to the front of the door, with ports fitting through holes cut in the mat. A safety temperature cut out was glued to the door underneath the mat. The temperature was controlled by a calibrated proportional temperature controller (RS Components Ltd) identical to that used for the jacket. The controller sensed temperature from a thermocouple fitted in a pocket in the heating mat. The controller was calibrated against a digital thermometer (Bibby Ltd.) attached to the inside of the door to ensure that the inner door temperature is that set on the controller.

This modification appeared to reduce condensation occurring on the door, but some was still found to collect in the downward facing door ports. To prevent this, the Miniclave 80 was fitted into a framework which tilted the machine backwards at an angle of 30° until the bottom ports were level allowing any condensate to run back into the chamber drain.

4.2.5 Modification of the Formalin Injection System

The original formalin injection system consisted of a Timer T2 controlling a solenoid valve opening long enough to allow approximately 3 ml of formalin into the vaporiser. This was inadequate for experimental purposes as it was too coarse in the control of the volume of formalin injected. In a preliminary modification (Chinyanganya, 1989) this was exchanged for a "dead

volume" injection system consisting of a 10 ml syringe with a top and bottom detection probe connected to an Electra level device. The Electra-Level could detect where the level of formalin was in the syringe by detecting the conductance of the fluid when it made contact with either top, bottom or both probes. This controlled injection of formalin so that the amount injected (drawn by vacuum) into the chamber was determined by the distance between the probes.

This system suffered from several faults. The volume injected could not be very accurately measured as the rate at which the formalin was injected was too rapid for the electronics to shut the controlling solenoid valve on the Miniclave 80. This meant that the formalin level often went below the lower limit probe resulting in difficulty in obtaining consistant formaldehyde concentrations in the chamber. Also, preliminary studies by Hurrell *et al*, 1983 and Chinyanganya, 1989 had indicated that there was a continuous loss of formaldehyde out of the chamber during the cycle. This meant that to maintain a constant concentration of formaldehyde in the chamber, subsequent smaller injections of formalin at regular intervals would be required. Since this could not easily be achieved with the single syringe injection system a two-syringe formalin injection system was devised.

This consisted of two 5 ml glass syringes attached to a perspex board. Each had an electrical contact plate attached to the top of the plunger (see Plate 4.2 and), and a microswitch at the bottom of the plunger's travel. This allowed the volume injected to be controlled by the distance between the top contact screw and the bottom microswitch. The first syringe was set to deliver a loading volume to reach the operating concentration. Once this injection had occurred, the multipulse controller detected this and switched

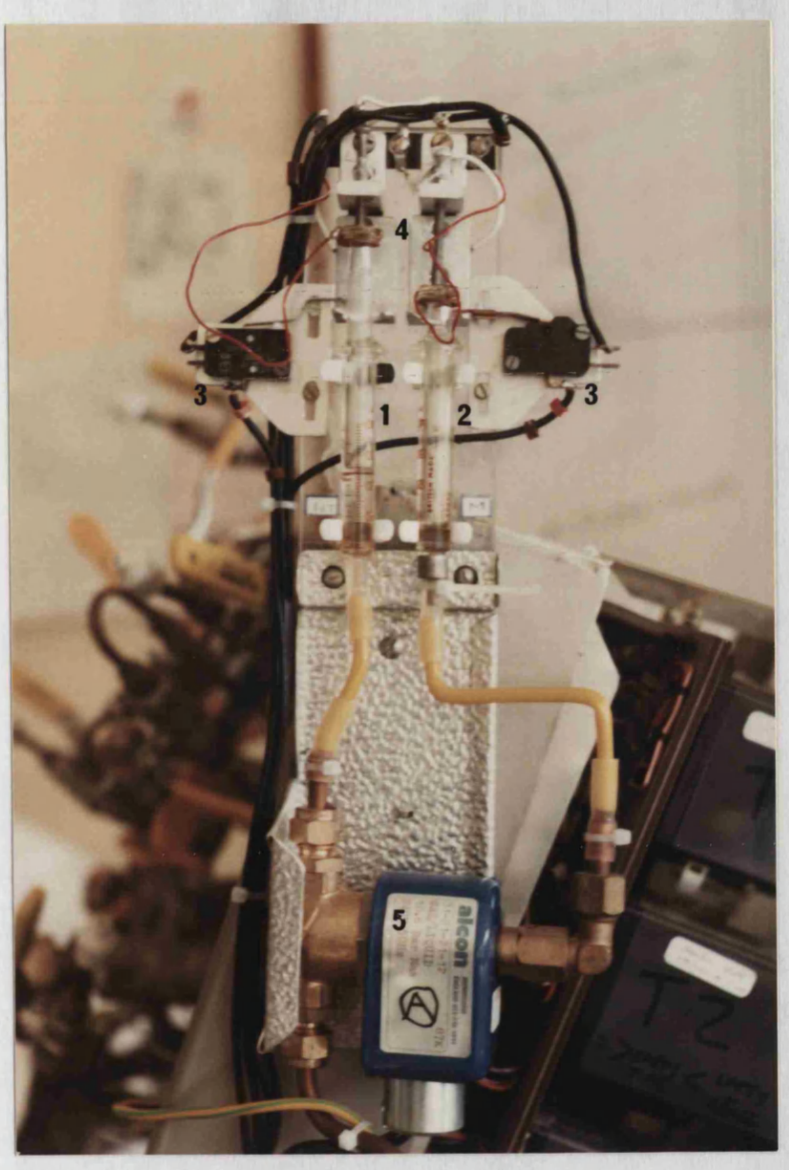


Plate 4.2 Photograph of the 'Two-syringe' Formalin Injection System

Illustrating : 1. First Pulse syringe

2. Multi-pulse syringe

3. Micro-switches to detect bottom of Plunger travel

4. Contacts to detect top of plunger travel

5. Valve X (section 4.2.5.1)

control over to the second syringe. This was set to deliver a smaller "top-up" volume. The same principle is used in the design of some constant drug delivery systems (Clark and Smith, 1981). Once the second injection had occurred, detected by the microswitch the injection was halted and the syringe was refilled with formalin from the reservoir to prepare for the next "top-up" injection.

4.2.5.1 Operation of the Control Circuit of the Modified Formalin Injection System

First pulse

A diagram of the control circuit for the two-syringe formalin injection system is illustrated in Figure 4.5. When the multi-switch on the Miniclave 80 reaches position 13 (start of formalin injection), a signal is taken from timer T2 supply to energize the Electra level (EL). When T2 is active, a signal taken from T2 energizes relay RA, (switching A and B down), to switch the Electra Level sensing probes (EL 7 and 8) to the pulse syringe. Valves X and Z are then energized allowing formalin from the first pulse syringe to be withdrawn into the vaporiser. Once the syringe empties, it switches microswitch C, which signals the close of valve Z and opening valve Y. This prevents further formalin entering the vaporiser, and allows formalin from the reservoir to top up the first pulse syringe. The formalin timer (T2) is adjusted to "time out" just as the first pulse syringe refills, to prevent reopening valve Z and allowing a second injection from this syringe.

"Top-up" pulse The Miniclave 80 multi-switch remains in position 13, and relay RA is de-energized (switching A and B up), transferring

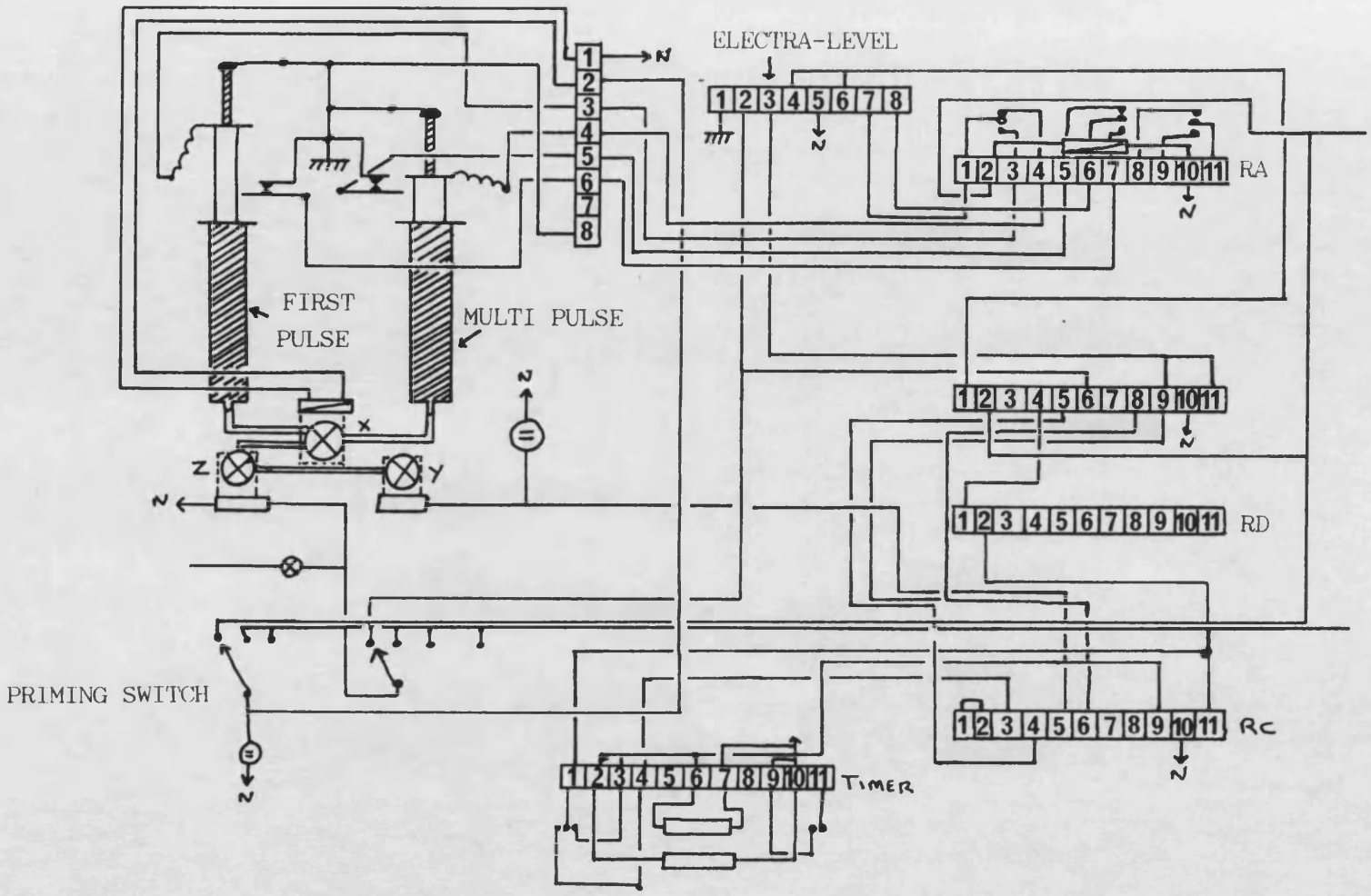


Figure 4.5 Circuit Diagram for the Operation of the 'Two-syringe' Formalin Injection System

(based on sketches by Line, S.J.)

control of the Electra Level sensing probes to the multi-pulse syringe. The supply to valve X is removed, shutting it and allowing formalin to fill and empty from the multi-pulse syringe. Once the Miniclave 80 reaches "temperature attained", the multiple formalin injection timer is energized (the timing mechanism can be set from 0-60 on a scale of seconds, minutes or hours). Each time this timer "times out", valve Z opens allowing formalin to be drawn into the vaporiser from the multi-pulse syringe. As the plunger of the syringe switches multiswitch D, valve Z closes and valve B opens refilling this syringe. As the plunger reaches the top of its travel, this is detected by the contact made, closing valve Y and restarting the multi-pulse timer. This cycle is repeated until the "hold period" timer (T1) times out, switching the Miniclave 80 multiswitch to position 14 for the elution stage.

4.2.6 Fitting of a Sampling Septum to the Rear of the Chamber.

To monitor the effectiveness of the injection system, samples of the chamber atmosphere needed to be taken and analysed for formaldehyde concentration. For this purpose, a brass fitting containing an industrial sampling septum (CMI Ltd.) was fitted to the rear of the chamber (Plate 4.4). A 12 inch, 20 gauge needle with luer lock fitting was inserted through this septum to approximately half way down the chamber. This allowed gas samples to be taken from the chamber in the vicinity of the test pieces when these were present in the chamber. A diagram of the Miniclave 80 chamber illustrating the modifications described is shown in Figure 4.6, and a modified piping diagram in Figure 4.7.

Plate 4.3 Photograph Illustrating the Brass Sampling Septum Attached to the Rear of the Miniclave 80 Chamber.

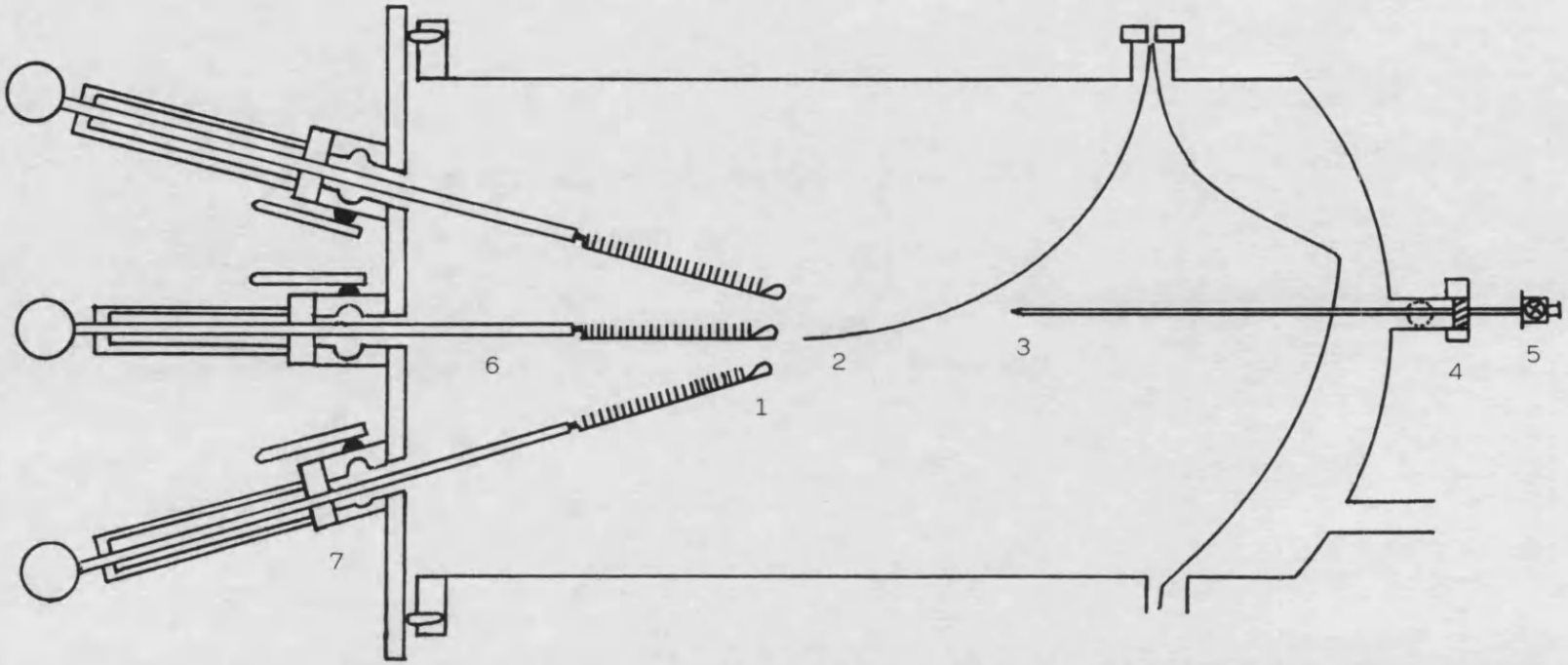


Figure 4.6 Illustration of the Modified Miniclave 80 Showing the Sampling Ports and Sampling Septum.

Key : 1. Test Piece Holders 2. Thermocouples 3. 12", 20 gauge needle 4. Sampling septum
5. Leur lock 6. Operating rods 7. Sampling ports and valves

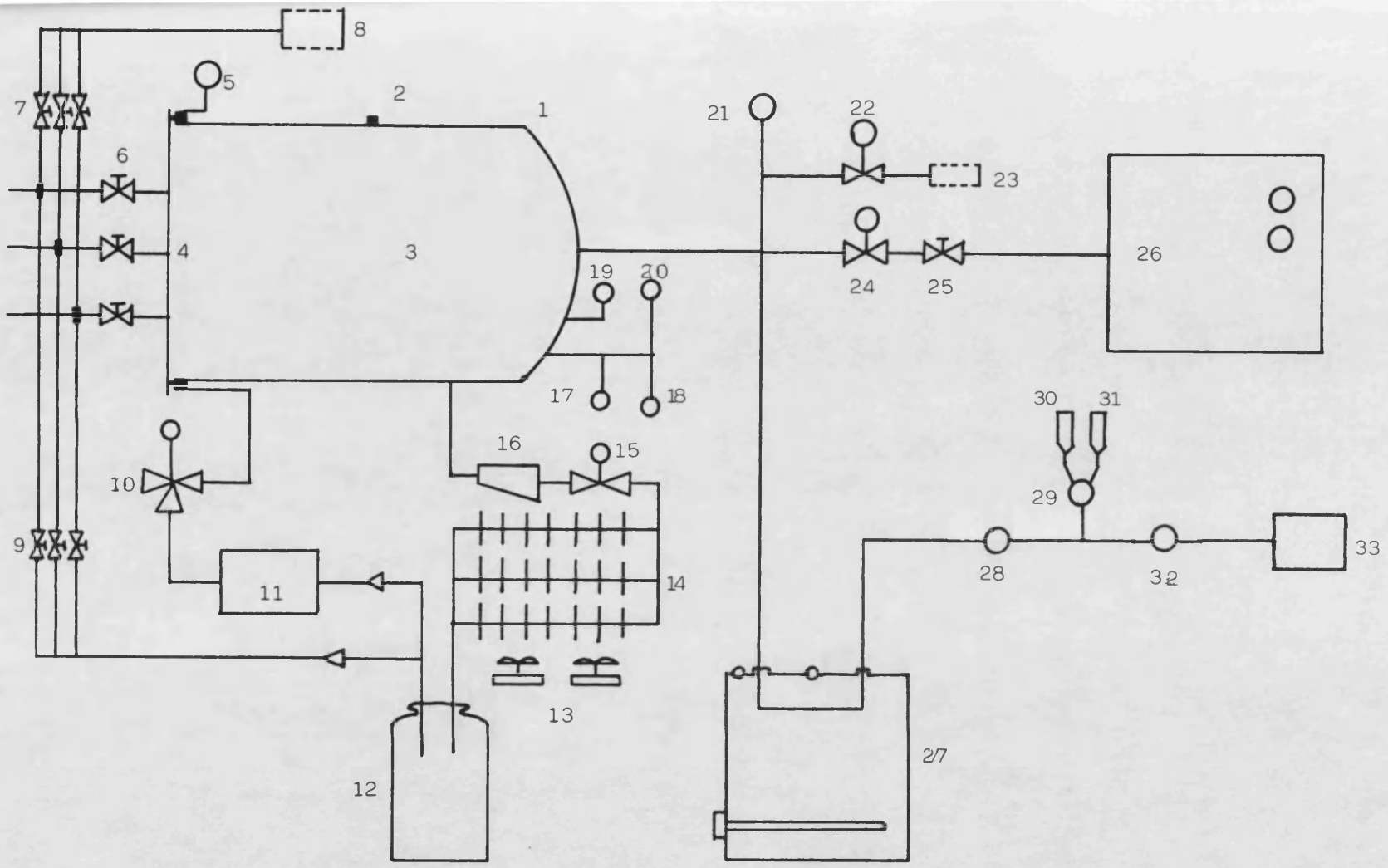


Figure 4.7 Piping Diagram of the Modified Miniclave 80 Illustrating the Sampling Ports and Two-syringe Formalin Injection System. (Developed from a diagram by Line, 1988)

Key to Figure 4.7 Piping diagram of the Modified Miniclave 80 Illustrating the Sampling Ports and Two-syringe Formalin Injection System.

1. Chamber heating jacket
2. Thermocouple entry port
3. Miniclave chamber
4. Miniclave door
5. Door seal pressure switch
6. Door valves for sampling ports
7. Air inlet valves for sampling ports
8. Air filter
9. Vacuum valves for sampling ports
10. Three way exhaust valve
11. Vacuum pump
12. Vacuum trap bottle
13. Cooling fans
14. Heat exchanger
15. Drain solonoid valve
16. Strainer
17. Vacuum switches
18. Vacuum gauge
19. Temperature thermostat and cut out switch
20. Temperatre gauge
21. Vacuum control switch
22. Air inlet control solonoid
23. Air filter
24. Steam inlet solonoid
25. Steam inlet control valve
26. Modified "Little Sister" Autoclave
27. Formalin vapouriser
28. Formalin inlet solonoid
29. Syringe selection solonoid
30. First pulse syringe
31. Multi-pulse syringe
32. Formalin from reservoir solonoid valve
33. Pressurised formalin reservoir

4.2.7 Demonstration of the Effect of the Modifications to the Miniclave 80 on the Ability of the Chamber to Hold a Vacuum

To demonstrate that the modifications carried out to the Miniclave 80 had not impaired the chamber's ability to hold a vacuum, it was necessary to carry out a "leak test". To perform this test, the machine was allowed to pull a vacuum of approximately 800 mbar gauge just prior to the first injection of formaldehyde. The power to the vacuum pump and cooling fans was then switched off. The increase in chamber pressure as atmospheric air leaked in was recorded over a 10 minute period. This procedure was first carried out with the original unmodified chamber door in place. It was then repeated with the modified door with the 3/4 inch valves shut, and with the 3/4 inch valves open and the secondary chambers attached. Finally the test was carried out with the modified door with the valves open and secondary chambers attached after the formaldehyde injection and sampling systems were fitted. The results of these tests are shown in Table 4.1. With all the modifications carried out, a maximum leak rate of 20 mbar over 10 minutes was recorded. This is 5 mbar over that recorded for the original unmodified machine. Though this represents a 33% increase, this was considered unavoidable due to leakage past the sampling rods in the secondary chamber. This increase was considered unlikely to seriously affect the operation of the Miniclave 80 and therefore was considered acceptable.

4.2.8 Operation of Modified Miniclave 80 to Expose Test Pieces of *B. stearothermophilus* Spores to LTSF Conditions

1. The Cal 9000 temperature controller was adjusted to the required operating temperature. The door and jacket temperature controllers were adjusted 2 degrees below that set on the Cal 9000.

The door and jacket were allowed 15 minutes to equilibrate at the set temperature.

Table 4.1 Data illustrating the Leak Rates of the Unmodified and Modified Miniclave 80.

Machine Setup	Leak Rate Over 10 Min
Original Machine	15 mbar
Modified Door 3/4" Valves Closed	16 mbar
Modified Door 3/4" Valves Open and Secondary Chambers Attached	20 mbar
3/4" Valves Open, Secondary Chambers Attached and Formalin Injection and Sampling Modifications Carried Out	20 mbar

2. The two-syringe formalin injection system was primed and adjusted as follows :- The screws on top of the syringes were adjusted to allow the travel of the plunger for a set volume. The multi-pulse timer switch was then set to position 1, allowing the first syringe to fill up to the stop. The switch was then set to position M, allowing the second syringe to fill. When both syringes were full, the switch was turned off. The seconds scale on the multipulse-timer was selected. The correct setting for the multipulse-timer to achieve the desired concentration in the Miniclave 80 chamber was found by experiment. The setting was different for each different

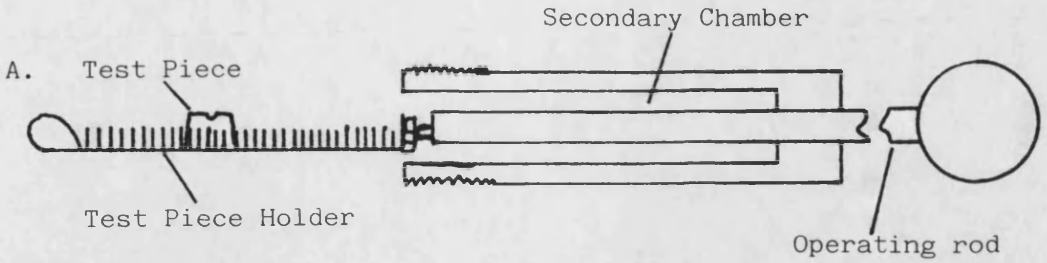
chamber temperature or concentration required.

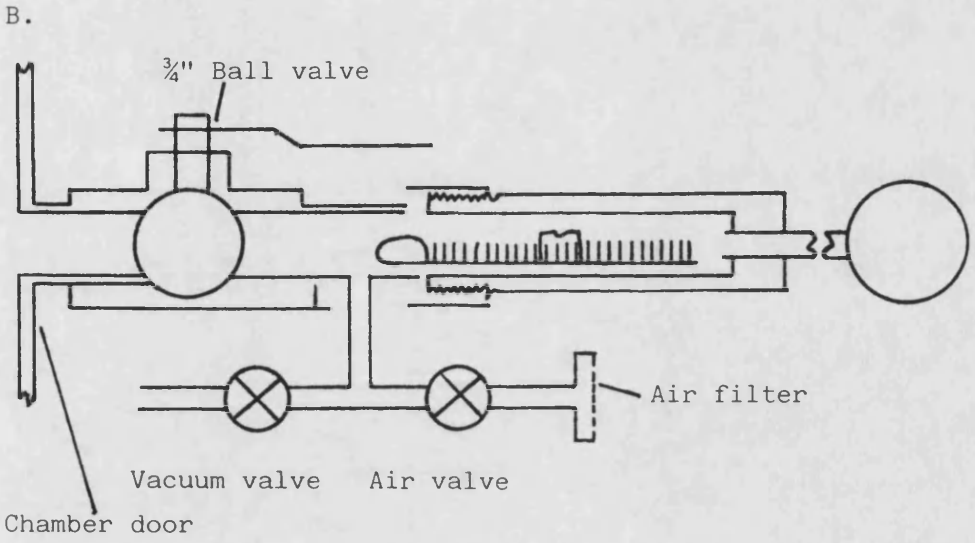
3. Test pieces, prepared as described in section 4.3.2, were loaded into the sample holder. The rod was then pulled to withdraw the samples into the secondary chamber. The orientation of the test pieces was noted.

4. All of the valves in the Miniclave 80 door were closed, the "Little Sister" Autoclave filled with water and a head of steam built up. The cycle of the Miniclave 80 was started, and allowed to proceed to the formalin injection stage, and the primary injection completed. The multi-pulse injections were then commenced, and allowed to proceed for three minutes to enable the formalin concentration in the chamber to stabilize.

5. Before, during and after the introduction of the test pieces into the chamber, samples of the chamber gas were taken as described in section 4.3.1.2, and assayed for formaldehyde concentration. The samples were chosen to ascertain the concentration just prior to, just after and in the middle of the individual formalin "top-up" injections. This gave estimations of the maximum and minimum concentrations attained, so an average could be calculated.

6. Test pieces were introduced into the Miniclave 80 using the sequence depicted in Figure 4.8 (A-E). The samples were held in the chamber for predetermined time intervals, and withdrawn as described in Figure 4.8 (F). Samples were only taken during the "hold" period of the cycle, the last being taken before the start of the elution stage.





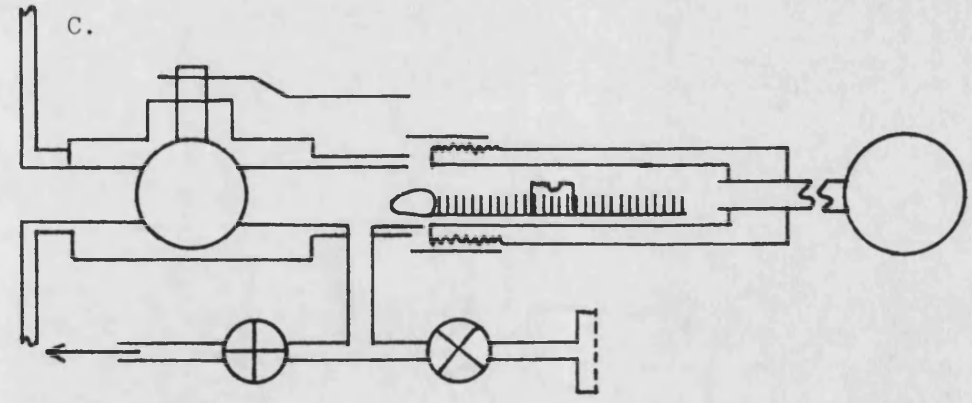


Figure 4.8 Chamber sampling system operating sequence (after Line, 1988)

A. Specimen holding device loaded with specimen cup

B. Specimen holding device withdrawn into secondary chamber, screwed onto ¾" ball valve, all valves ALL valves closed

C. Vacuum valve opened for 60 seconds to evacuate secondary chamber

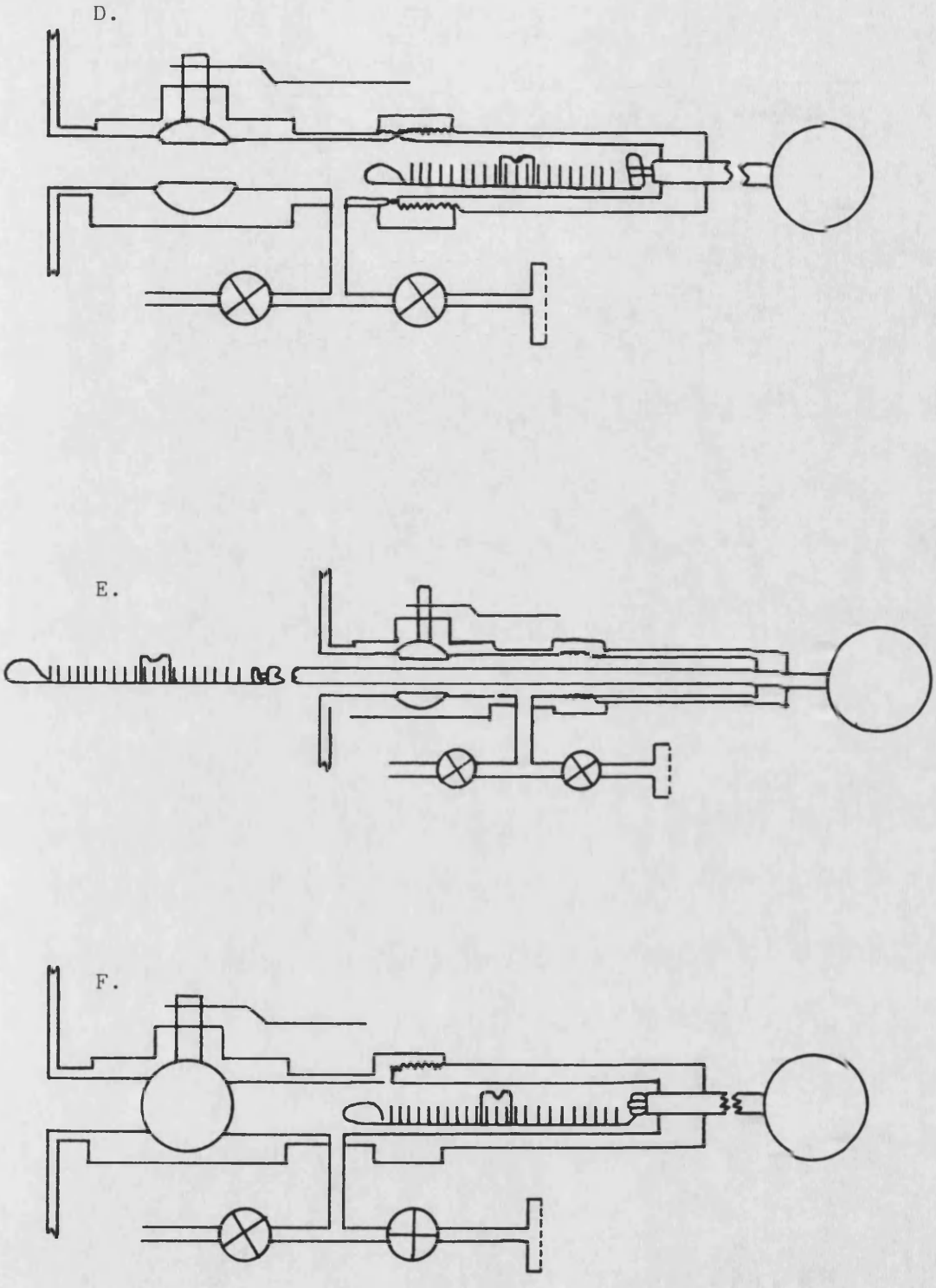


Figure 4.8 Chamber sampling system operating sequence (after Line, 1988)

D. Vacuum valve closed, ¾" valve opened and timing started

E. Test piece holder and test piece introduced into chamber

F. On completion of LTSF exposure, test piece withdrawn into secondary chamber, ¾" valve closed and air valve opened, secondary chamber then removed and test piece transferred to 1% glycine solution

7. At the end of a sterilization cycle, the "elution" indicator is illuminated, and elution and return to atmospheric pressure occurs the same as in the unmodified Miniclave 80 as described in section 4.1.1.

4.3 EXPERIMENTAL

4.3.1 Demonstration of the Effectiveness of the Two-Syringe Formalin Injection System

Experiments were carried out to demonstrate the improvement gained from the new injection system. First, a single pulse injection of 1 ml was used, with no secondary injection. This was to simulate the conditions used in the study reported by Chinyanganya (1989). Secondly, an initial pulse of formaldehyde followed by smaller secondary pulses was used to give an approximate formalin concentration of 12 µg/ml.

4.3.1.1 Development of the Chamber Gas Sampling System

To assay the formaldehyde in the Miniclave 80 chamber, it was necessary to first withdraw a gas sample from the chamber under operating conditions (approx. 73°C, 0.6 bar). This was achieved using a syringe attached to the sampling system described in section 4.2.6. The ideal choice of syringe would have been Hamilton gas syringes, but as it was desirable to take as many gas samples as possible during a cycle, and as each sample took 72 minutes to process, this would have been very costly. It was decided therefore, to use standard 30ml volume syringes of either glass or plastic construction. The plungers of the glass syringes were coated with a thin layer of silicone vacuum grease. This would be required if they were to be used for sampling gas from the Miniclave 80 chamber whilst

it was under vacuum. The plastic syringes have no such need as they have a rubber end to the plunger, which forms a good airtight seal.

To compare the effectiveness of glass and plastic syringes, it was necessary for two syringes, one glass and one plastic to simultaneously sample a gas mixture containing formaldehyde.

The gas jar, was constructed of 3mm thick Pyrex glass to prevent explosion. Approximately 1 ml of formaldehyde solution was put into the glass jar, and the top sealed with a metal cap with a rubber septum. A 20 gauge needle was inserted through this septum, and to the other end, a two way valve and Y-connector were fitted.

With the two way valve in the closed position, the glass jar was lowered into an oil bath at 110°C. This caused the formaldehyde solution to vaporise and form a mixture of steam and formaldehyde gas as would be found in the Miniclave 80 chamber. This was left to equilibrate for 20 minutes. After equilibration, two of the syringes under test were attached, one to each end of the Y-connector. The two way tap was then opened, and the positive pressure in the jar drove formaldehyde gas into both syringes simultaneously. When both syringes contained a pre-determined volume of gas the tap was closed. The syringes were carefully removed from the Y-connector to prevent movement of the plunger and consequent loss of sample.

25 ml Volumes of a 0.05% MBTH solution were then drawn into the syringes. The syringes were then agitated to mix the gas and the MBTH solution together, and left to stand with occasional agitation for 1 hour. After this time, a 10 ml volume from each syringe was dispensed into test tubes. To each tube, 2 ml of the oxidising solution was added, the tube vortexed, then left to stand for 12 minutes. The absorbance at 628 nm of the solution in each tube was then read using a spectrophotometer.

This procedure was first carried out using one glass and one plastic syringe. The results of this experiment are shown in Table 4.2. The concentration per ml of formaldehyde was calculated using the Molar Extinction Coefficient (E) as described in section 2.3.2.1. This data clearly illustrates that the plastic syringes gave a consistantly lower estimate of the concentration of formaldehyde than the glass syringes. During an experiment to expose spores to LTSF, the gas samples taken during the experiment were commonly assayed using 25 ml of 0.05% MBTH solution. With a typical gas sample of 5 ml, the difference in estimation of the concentration of formaldehyde from the absorbance obtained would be five times the estimated concentration per ml listed in Table 4.2. For comparison this experiment was repeated using two glass syringes, the plunger of one had been coated in a thin layer of silicone vacuum grease and the second had not. These syringes exhibited a much more reproducible estimate of formaldehyde concentration as shown by the differences in absorbances and formaldehyde concentrations recorded in Table 4.3. The differences in formaldehyde concentration were calculated as for Table 4.2. This data also demonstrates that the coating of silicone grease on the plunger of one of the syringes does not affect the reproducibility of the estimates using glass syringes.

On the basis of these results, glass syringes with silicone grease coated plungers were used for all further formaldehyde gas assay work.

4.3.1.2 Comparison of Single Syringe and Two-syringe Formalin Injection Systems

Once that all the modifications of the Miniclave 80 had been completed, it was necessary to use the gas sampling system chosen to

Table 4.2 Comparison of Glass (unlubricated) and Plastic Syringes for Use in Estimating Formaldehyde Concentration in Gas Samples by The MBTH Method

Absorbance at 628 nm		Difference in	Difference in
Glass Syringe	Plastic Syringe	Absorbance	Estimate (mg/l)*
0.369	0.143	0.226	0.231
0.359	0.195	0.164	0.168
0.332	0.178	0.154	0.158
0.562	0.493	0.069	0.071
0.489	0.384	0.873	0.893
0.523	0.259	0.782	0.800
1.884	1.269	0.615	0.629
0.373	0.307	0.066	0.068

* Calculated using the Molar Extinction Coefficient. In a "typical" gas assay from the Miniclave 80 (25 ml MBTH, 5 ml Sample), the difference in estimate of formaldehyde concentration in the chamber would be five times the amount calculated in this column.

Table 4.3 Comparison of Glass (unlubricated) and Glass (lubricated) Syringes for Use in Estimating Formaldehyde Concentration in Gas Samples by The MBTH Method

Absorbance at 628 nm		Difference in	Difference in
Glass (unlub)	Glass (lub)	Absorbance	Estimate (mg/l)*
1.842	1.790	0.052	0.053
1.031	0.997	0.034	0.035
0.315	0.349	0.034	0.035
0.991	0.886	0.105	0.107
0.494	0.530	0.036	0.037
0.402	0.373	0.029	0.030
0.807	0.745	0.062	0.063
0.110	0.136	0.026	0.027
1.699	1.690	0.009	0.009
1.704	1.682	0.220	0.225

* Calculated using the Molar Extinction Coefficient (E). In a "typical" gas assay from the Miniclave 80 (25 ml MBTH, 5 ml Sample), the difference in estimate of formaldehyde concentration in the chamber would be five times the amount calculated in this column.

assess the effectiveness of the two-syringe injection system. It was also decided to compare this with the single injection as used by Chinyanganya (1989).

For both experiments the Miniclave 80 cycle temperature controller was set to operate at 73°C and the jacket and door controllers to 71°C. Also the 1st syringe was set to deliver a 1 ml injection of formalin to the vaporiser. In the first experiment the second syringe, used to deliver the "top-up" injection was switched off. This would then simulate the single 1 ml injection of formaldehyde used by Chinyanganya. In the second experiment, the second syringe was set to 0.2 ml injection, and the timer that controlled the intervals of injection of this syringe to 1.5 minutes.

To assess the effectiveness of the single injection, gas samples were taken from the sampling septum over a 30 minute period. This was achieved by drawing a 5 ml gas sample into a glass syringe which contained 25 ml of 0.05% MBTH solution. Nine gas samples were taken in total, the ends of the syringes were then sealed, and the concentration of formaldehyde assayed as described in 4.3.1.1. The results of this experiment are shown in Figure 4.9.

To demonstrate the improvement gained (if any) by the use of a "top-up" volume, this experiment was repeated with the second syringe set up to inject 0.2 ml of formalin every 1.5 minutes. Gas samples were taken over a 10 minute period with more frequent sampling than in the previous experiment. The results of this experiment is illustrated in Figure 4.10.

The results illustrated in Figure 4.9 demonstrate that in a single pulse cycle, 90% of the formalin which entered the chamber atmosphere at the initial pulse was lost in just 3 minutes. The results in Figure 4.10 show that with the dual injection system, the

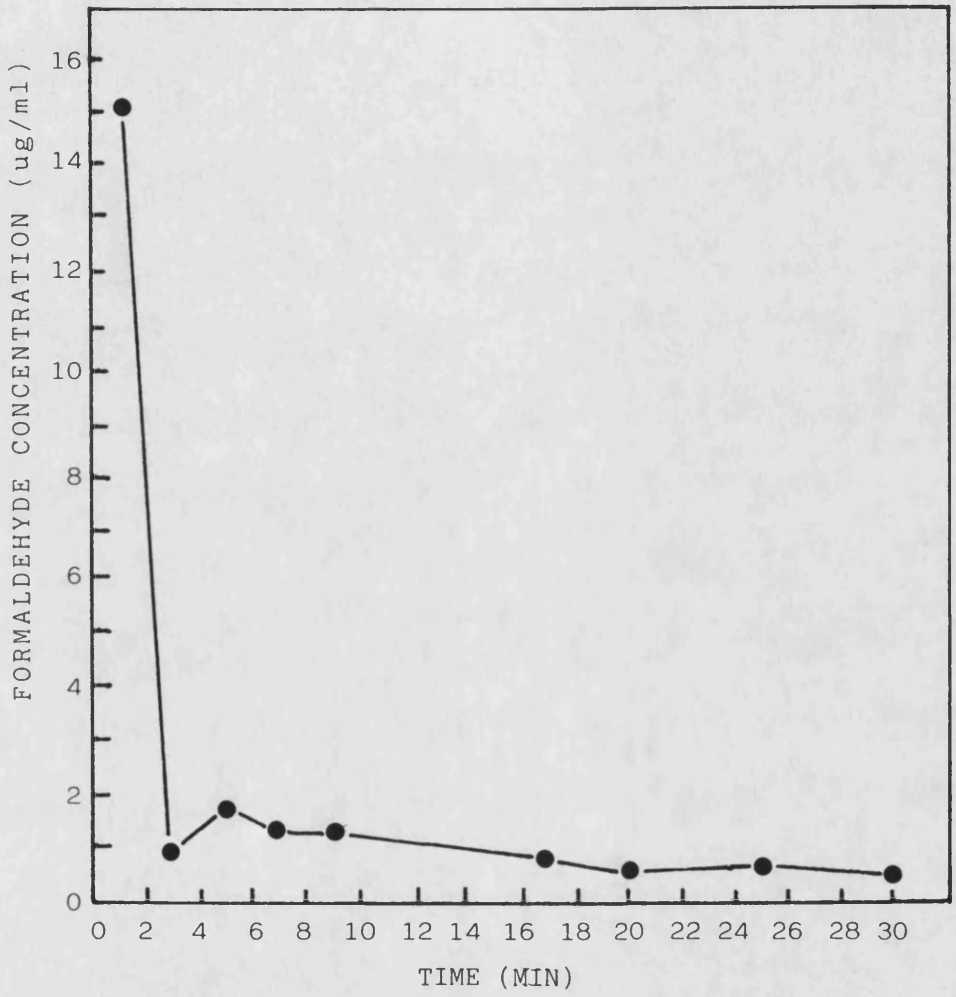


Figure 4.9 Concentration of Formaldehyde in the Chamber of the Miniclave 80 at Time t after a Single 1 ml Injection of 38% w/v Formalin at an Operating Temperature of 73°C.

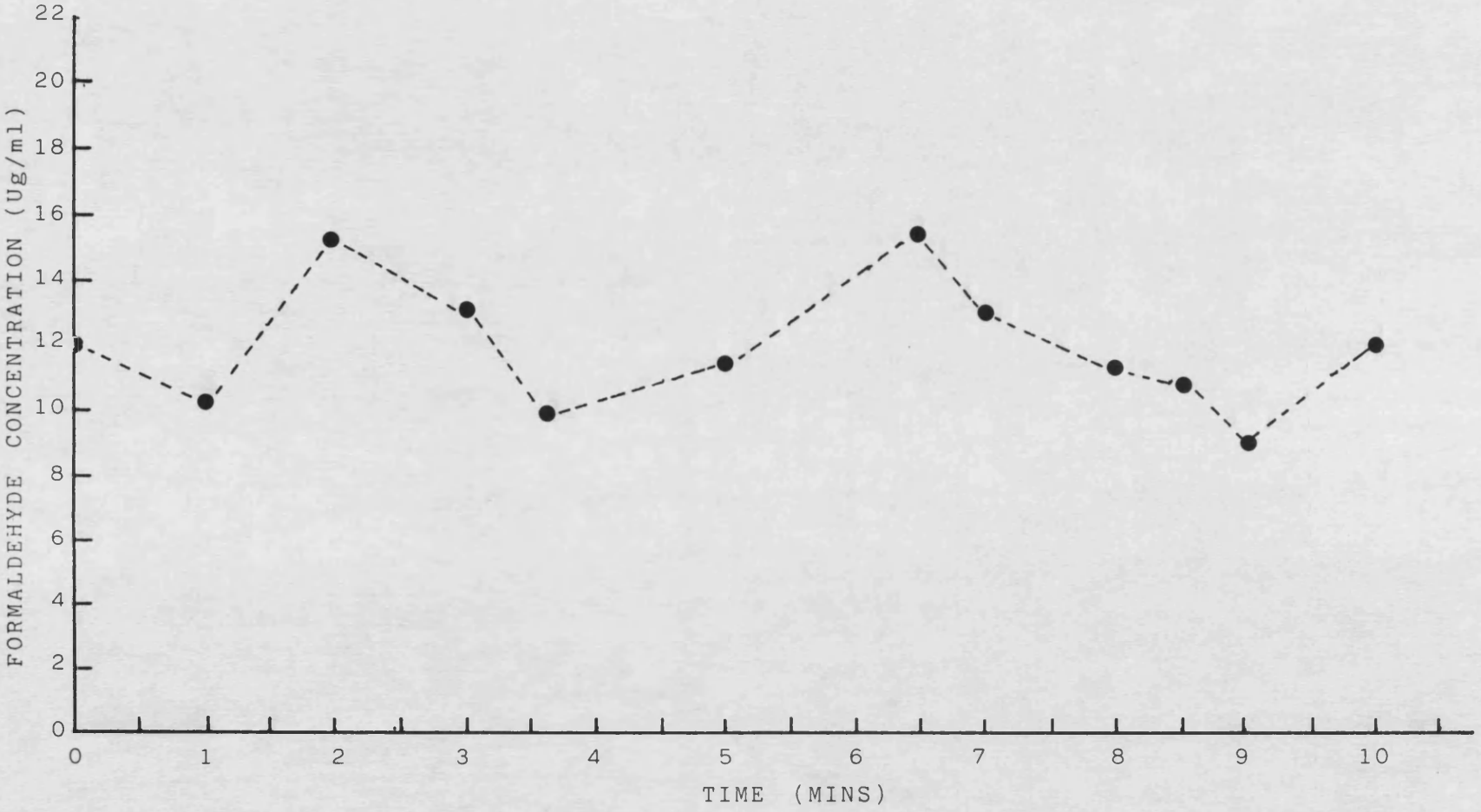


Figure 4.10 Formaldehyde Concentration in the Miniclave 80 Chamber Using the 'Two-syringe' Formalin Injection System (0.2 ml every 1.5 min). First Gas Sample Taken 3 min After Initial Injection.

formaldehyde concentration can be set to a required level (by experimentation) and can be maintained at a level of 12 µg/ml ± 3µg/ml. These limits reflect inevitable cycle fluctuations (temperature and pressure variations) and uneven mixing of the chamber atmosphere. Gas sampling times were chosen so as to reflect the concentration of formaldehyde just after, midway and just prior to each of the second syringe injections. The intention was to demonstrate the range of concentrations that occurred in the Miniclave 80 chamber. Therefore the level and range of concentrations obtained in this experiment should reflect the maximum and minimum concentrations in the cycle.

The conclusions that can be drawn from these studies are that the single formalin injection system is of no practical use for the production of survivor curves, as the formaldehyde concentration is not constant. The dual formalin injection system maintains a better, more constant level of formaldehyde in the chamber. Although this level is not perfectly stable, it is accepted that this is due to limitations set by the inevitable fluctuation of conditions in the chamber, and therefore was considered suitable for use in further studies.

4.3.2 Preparation of Test Pieces

Once the Miniclave 80 had been shown to be acceptable as a test apparatus, it was necessary to introduce test pieces upon which spores of the organism to exposed to LTSF were deposited.

Aluminium carriers (Albert Browne Ltd, Leicester) were used to carry the spores for exposure to LTSF conditions. The carriers were supplied as 20 mm x 30 mm x 0.1 mm aluminium foil, with a 6 mm

diameter well in the centre. These were cut down to approximately 15 mm x 8 mm (length x width), and the ends bent down at right angles to provide legs (Figure 4.11 to fit into the test piece holder Figure 4.4

The carriers were cleaned of grease and dirt by soaking in a dilute solution of Linkdet 704 (Link Chemicals Ltd) overnight. This was followed by five washes in tap water, then five in glass distilled water. The cleaned carriers were then soaked in acetone for a minimum of three hours with occasional stirring. They were then allowed to dry, placed in clean glass petri-dishes, and sterilized at 160°C for a minimum of 1 h.

4.3.2.1 Inoculation of Carriers

The sterile carriers were aseptically placed, legs down, into the surface of plain agar (Oxoid Number 3), under laminar flow conditions. Aliquots of 20 µl of spore suspension, containing approximately 1 x 10^6 spores (determined by viable count as described in Section 2.3.1.3), were then loaded into the well of each carrier. These were then allowed to dry under laminar flow for 4 h at approximately 25°C, (relative humidity was not controlled). The loaded carriers (test pieces) were stored for a maximum of 24 h at 4°C in the dark prior to use.

4.3.2.2 Removal and Determination of Viable Count of Spores from Test Pieces

In order to assess the resistance of spores to LTSF conditions, it would be necessary to remove the spores from the test pieces. This would allow the enumeration of the survivors by traditional recovery

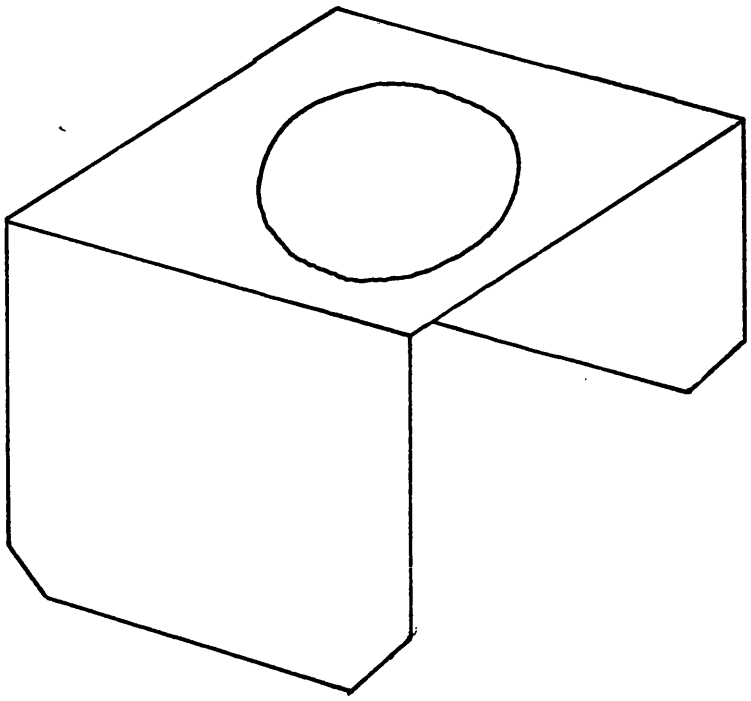


Figure 4.11 Illustration of an Aluminium Test Piece

techniques. Various methods have been used by previous workers for recovering spores off carriers, including homogenisers (Everall and Morris, 1978), Glass beads and simple agitation (Dewhurst *et al*, 1986)

The method of removal chosen was sonication at 51 kHz, as this had been shown to be the most effective (Dewhurst *et al*, 1986) and had been used successfully by Chinyanganya (1989). Before using the sonication bath, it was necessary to identify the nodes (strongest sonication points) in the bath. This was done by hanging sheets of aluminium foil (20cm x 20cm) from glass rods into the sonic bath. These sheets were suspended across the width of the bath with 1 cm spacing. Water with 1% Tween 80 was used to fill the bath. The sonicator was operated for 15 min, and each sheet of foil was examined for holes produced by the vibrations. The positions in the bath where the maximum damage to the foil occurred were noted. The bottles containing test pieces were suspended at these positions in the bath to ensure effective sonication.

4.3.2.3 Determination of Optimum Period of Sonication

It was necessary to determine the sonication time required to remove all the spores from the carrier, and to ascertain the effect of the treatment on the spores. Nine thin walled bottles, containing 10 ml of 1% glycine were each loaded with 1 test piece containing *B. stearothermophilus* NCIB 8224 spores. In addition, four bottles containing 9.98 ml of 1% glycine had 20 µl of spore suspension from the same batch added to them. Three bottles containing test pieces were sonicated for 5 min, three for 10 min and three for 15 min. The bottles containing spore suspensions were sonicated for a total of 15

min, with 1 ml samples taken after 0, 5, 10 and 15 min. Samples from all 12 bottles were serially diluted to 10^{-4}, and triplicate 0.2 ml volumes spread plated onto Nutrient Agar. These plates were incubated at 55°C for 5 days and the resultant colonies counted. The results of this study are shown in Table 4.4. A t-test was carried out on the average number of colonies after 10 minutes sonication and the contol after 0 minutes sonication. The value of 0.73 obtained (Table 4.5) indicating that it has attained the same count as the control, demonstrates that all the spores are recovered off the carrier in this time. Moreover, extending the sonication time to 15 minutes does not significantly increase this as shown by the t-test on the 10 and 15 minute sonication data (Table 4.5). To demonstrate that sonication has no effect on the viability of the spores, a t-test was carried out on the control 0 and 15 minute sample results. The result shown in Table 4.5 show that there is no significant difference, therefore sonication has no effect on germination and outgrowth of the spores. Finally, to demonstrate the reproducibility of this technique for removal of spores off the aluminium carriers, an analysis of variance was carried out on the data for colony counts after ten minutes sonication. The results shown in Table 4.6 show that there is no significant difference within or between replicate counts, and with a coefficient of variation of 5.0%, is considered suitable for use in further studies.

Table 4.4 Determination of Optimum Period of Sonication of Test Pieces to Remove all the Spores of *B. stearothermophilus* NCIB 8224, and its Effect on Spore Viability

Samples	Time (min)	Dilution	Counts per Plate	Mean
Test piece	5	10^{-4}	47 53 49 54 58 70 46 52 55	53.8
Test piece	10	10^{-4}	87 91 85 89 87 91 97 86 85	88.7
Test piece	15	10^{-4}	84 86 89 85 80 82 83 83 92	84.9
Control	0	10^{-4}	91 95 94 89 87	91.2
Control	5	10^{-4}	97 89 95 85 96	92.4
Control	10	10^{-4}	86 86 97 88 91	89.6
Control	15	10^{-4}	91 96 92 84 88	90.2

Table 4.5. Students t-test Analysis of the Data Presented in Table 4.4

Variable 1	Variable 2	t-value	Degrees of freedom	Probability of occurence
0 min Control	10 min treatment	0.73	14	$P > 0.1$
10 min treatment	15 min treatment	1.064	18	$P > 0.1$
0 min control	15 min contol	0.28	10	$P > 0.1$

Table 4.6 Two-way ANOVA of Numbers of Spores Recovered From Test Pieces of *B. stearothermophilus* NCIB 8224 by Sonication

Sample	Colony counts per plate	Mean count
1	87 91 85	87.7
2	89 87 91	89.0
3	97 86 85	89.3

Analysis of Variance

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Between Samples	2	36.222	18.111	0.9
Within Samples	2	1.5556	0.7778	0.0
Residual	4	79.111	19.778	
Total	8	116.889		

Coefficient of Variation = 5.0%

$F(4, 2)\ P_{0.05} = 19.25$

4.3.3 Exposure of Spores of *B. stearothermophilus* NCIB 8224 Sporulated on C-LTD Medium to LTSF

Before studies on the effect of variations in either concentration of formaldehyde or temperature, it was necessary to demonstrate the reproducibility of survivor curves produced using the modified Miniclave 80.

For this test pieces of spores of *B. stearothermophilus* sporulated on C-limited medium were prepared as described in 4.3.2. These test pieces were then aseptically placed into the test piece holder on the sampling rods as illustrated in Figure 4.3. The test pieces on the holder were withdrawn into the secondary chambers and then attached to the 3/4" ball valves on the Miniclave 80 door as in Figure 4.8 (A-B). The Miniclave 80 controls were adjusted, and the test pieces introduced into the chamber and removed from the chamber as described in section 4.2.8. Once the test pieces were removed from the chamber, they were removed from the test piece holders and put individually into thin walled glass bottles containing 1% w/v glycine solution. This solution inactivates any formaldehyde carried over on the test pieces, and has been demonstrated to have no effect on the viability of spores of *B. stearothermophilus* (Chinyanganya, 1989). These bottles were then suspended in a sonic bath and sonicated for 10 minutes and the number of viable spores enumerated as described in section 4.3.2

4.3.3.1 Treatment of Data Obtained from LTSF Exposure Experiments

The plot of the three survivor curves obtained from the experiment in section 4.3.3 are illustrated in Figure 4.12. As all three sets

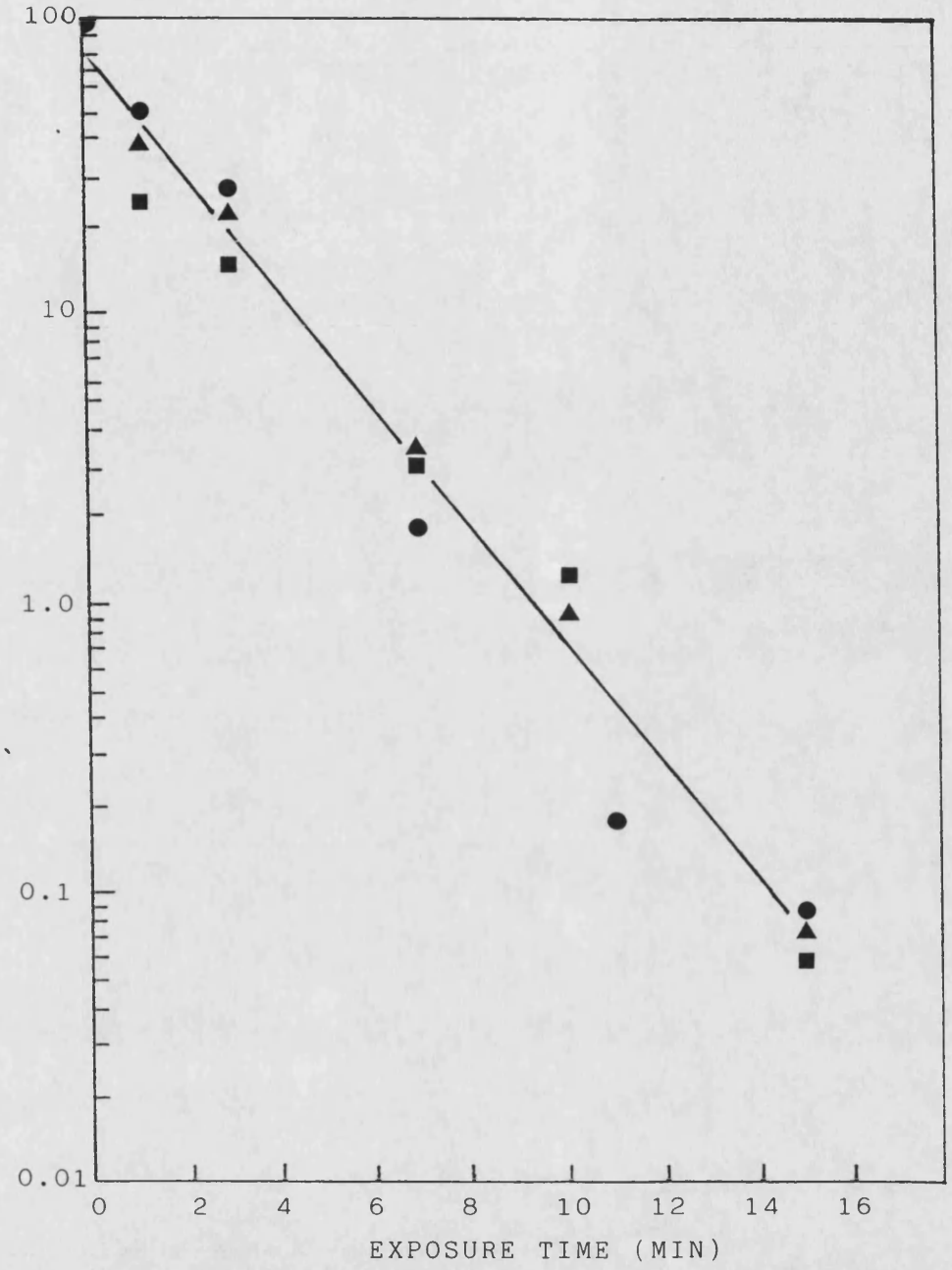


Figure 4.12 Plot of Three Replicate Survivor Curves of Spores of B. stearothermophilus NCIB 8224 Produced on C-limited Medium When Exposed to LTSF at 73°C and 12 ug/ml Formaldehyde Concentration.

of data demonstrate a linear trend, they can be analysed by Least Square Regression Analysis, as described in section 3.3.5.

Regression analysis of the data for the three survivor curves illustrated in Figure 4.12 was carried out. This yields two useful attributes of the data, the intercept on the dependant variable axis and the slope of the line. These values, plus their standard errors and variances (calculated as described in Appendix III) are recorded in Table 4.7. Assuming that they are straight line models, to demonstrate that they are all the same straight line, a student's t-test analysis of the slopes and intercepts was carried out. Each of the intercepts and slopes were compared in pairs in each of the three possible combinations (i.e. 1 & 2, 1 & 3 and 2 & 3). The results of these analyses are recorded in Table 4.7.

The results of this analysis demonstrates that there is no significant difference between either the slopes or intercepts of the three survivor curves, that is all the calculated t-values are less than the significant t value for 6 degrees of freedom (t(6)) at p=0.05. This illustrates the reproducibility of the technique, and it was therefore considered suitable for use in further studies

4.4 DISCUSSION

During the work reported in this chapter, many modifications were carried out to the Miniclave 80. After completion of these, it was necessary to demonstrate the machine's suitability for use in studies on the effect of exposure of spores of *B. stearothermophilus* NCIB 8224 to various conditions of LTSF.

This suitability was demonstrated in various ways. Firstly the leak test carried out in section 4.2.7 demonstrated the ability of

Table 4.7 T-test to compare Slopes and Intercepts Obtained by Linear Regression Analysis of Three Replicate Survivor Curves of Spores of *B.stearothermophilus* NCIB 8224 Exposed to LTSF with 12µg/ml Formaldehyde at 73°C.

Exposure Time (min)	Log Percentage Survivors (1)	(2)	(3)
0	2.00	2.00	2.00
1	1.48	1.70	1.36
3	1.0	1.44	1.08
7	0.33	0.26	0.52
10	-0.01	-0.80	-0.15
15	-1.22	-1.15	-1.22

Regression Data

Replicate	Slope	Standard Error	Variance	Intercept	Standard Error	Variance
1	-0.1899	0.0180	0.0016	1.788	0.144	0.104
2	-0.2269	0.0233	0.0027	1.936	0.186	0.174
3	-0.1969	0.0141	0.00098	1.779	0.113	0.064

t-test

Comparison	t-value (slope)	t-value (intercept)	Degrees of Freedom	t (6) for p = 0.05
1 and 2	0.56	0.28	6	2.45
1 and 3	0.139	0.022	6	2.45
2 and 3	0.49	0.32	6	2.45

the modified chamber to hold a vacuum when compared to the unmodified chamber. The increase in leakage was up to 33%, but was considered unavoidable, and unlikely to have detrimental effects on the Miniclave 80 operation. Secondly, the improvement on the single formalin injection system obtained by the fitting of a two-syringe injection system. This involved the choice of how to sample the gas of and selecting an assay methodology. To sample gas from the leur lock on the sampling septum, it was necessary to use a syringe. The choice of syringe (either plastic or glass) was made on the basis of a comparison of the reproducibility of estimates of formaldehyde concentration in a model system.

The results of the comparison of glass and plastic syringes are illustrated in Table 4.2. These results show that the plastic syringes gave a consistantly lower estimate of the formaldehyde concentration than that of the glass syringes. When this evidence was examined in the light of the results of a comparison of glass syringes, Table 4.3, it can be seen that glass syringes gave much more reproducible results even though the plunger of one was coated in vacuum grease and the other was not. In fact, the differences between two glass syringes and a glass and a plastic syringe show almost 10-fold lower differences in estimates. These data demonstrates that glass syringes give more reproducible results and that the addition of a vacuum grease (required to sample gas under vacuum) to the piston has little or no effect on the reproducibility, or the estimate as compared to an unlubricated glass syringe.

This reproducibility problem with plastic syringes is almost certainly due to some interaction between the plastic and either the formaldehyde, or the reagents themselves. However, despite this low

reproducibility, the plastic syringes would have been better suited to withdrawing gas samples out of the chamber under vacuum.

On the basis of the results discussed above, glass syringes (using minimal silicone vacuum grease) were used in all formaldehyde sampling experiments during modification and use of the Miniclave 80.

It is important to note that the formaldehyde assay experiments discussed above only demonstrate the reproducibility of the MBTH colorimetric assay using glass syringes. It does not demonstrate its accuracy, as this would not be possible to establish with the apparatus described in 4.3.1.1. To do this it would be necessary to have a second, proven method of formaldehyde gas measurement, with which to compare the estimates obtained by the MBTH assay. The only other way would be to calculate the theoretical concentration of formaldehyde in the jar, assuming total vaporisation of the volume of formalin put in. This was attempted, and was totally unsuccessful, almost certainly due to formaldehyde gas dissolving in condensate which formed on cooler parts of the jar, therefore giving lower estimates of concentrations than calculated.

The best way of determining its accuracy would be to prepare a gaseous standard similar to that used by Boutonnat *et al*, 1988, or using a Kinitek model 670 permeation tube system as used by Lipari and Swarin (1982). To calibrate the MBTH method by either of these gaseous methods would be preferable to the aqueous solution of formalin used to calibrate it in this study.

The study on the formalin injection control demonstrated several points. Firstly, the results illustrated in Fig 4.9 show that a single pulse of formaldehyde causes a peak in concentration of formaldehyde gas in the chamber. However, within a short time, the

majority of this is lost from the chamber. This result is in agreement with the findings of Chinyanganya, though he deduced it by indirect methods. This result is not unexpected as this effect has been reported previously (Marcos and Wiseman, 1979; Hurrell *et al*, 1983). Figure 4.10 depicts the improvement in maintaining a set formaldehyde concentration using the new multiple pulse method. It can be seen that peaks and troughs in level of concentration still exist. However, this is a distinct improvement on the result of the single pulse, and is probably as constant a level as it is feasible to obtain with this sort of system. With this system to control the formaldehyde concentration, the method in 4.3.1 to monitor it, and the improved temperature controls and steam supply, it is possible to control the conditions under which spores are inactivated to a high degree. These systems were employed successfully to generate the survival curve illustrated in Figure 4.12 and the data discussed in chapter 5 and 6.

Nevertheless, even with this drawback, the method of sampling and measurements of formaldehyde described in this chapter have been used successfully for the development of the multiple pulse injection system on the Miniclave 80. It has allowed the measurement of the drop in formaldehyde concentration which occurs in the Miniclave chamber during operation, and hence the amount of formaldehyde needed in subsequent injections to offset this loss. This resulted in the production of reproducible linear survivor curves described in Chapter 5. These curves exhibit a drop in survivors of up to 4 log cycles, the greatest achieved with this particular organism and apparatus, the best previously achieved was 1 log cycle (Chinyanganya, 1989).

There are two major drawbacks with this method of sampling and measurement of formaldehyde concentration. First is the physical difficulty of withdrawing a gas sample from a chamber under vacuum, using a syringe and operating a two way valve simultaneously whilst introducing and removing samples from the chamber as illustrated in Figure 4.8 (A-F). The second is that the whole system is a manual one, needing a minimum of 72 minutes from sample withdrawal to obtaining a result. This obviously does not lend itself to real time feedback needed to obtain a constant level of formaldehyde in the chamber, hence, even averaging out results for several cycles, there are still quite large fluctuations in the formaldehyde concentration (± 3 ug/ml).

The ideal method for use in this system would obtain results nearly instantaneously, preferably totally automatic, and giving the result in a current or voltage output proportional to the concentration of formaldehyde. This sort of method would allow the development of a feedback system, using a detector linked to a computer analog input. The computer could measure the current or voltage, and use this information to calculate the amount of formaldehyde needed to maintain a constant level, and inject it using a relay controlled solenoid valve.

Three methods which would allow the development of such a system have been described. The three use totally different approaches, one being based on pulsed laser optics, one on the frequency of vibration of a piezoelectric crystal, and finally one is an automated fluorometric assay.

The pulsed laser system reported by Boutonnat *et al*, (1988), is based on photoacoustic (PA) spectroscopy. This operates on the

principle of different compounds being excited by different wavelengths of light emitted from a pulsed dye laser. The absorption/excitation wavelength for formaldehyde is 303.59 nm, and this can be measured directly. It is necessary to know the absorption wavelengths of any other compounds present in the gas mixture (e.g. water, methanol) so that their concentrations can be measured, and their contribution to measurement at 303.59 nm can be calculated and subtracted. This method was used successfully to measure the concentration of formaldehyde in a gas mixture containing NO_2 and CH_3CHO (Boutonnat *et al*, 1988). There are two obvious drawbacks to this system. The first is that dye lasers are very expensive, and it would require three on the Miniclave to measure water, methanol and formaldehyde concentrations. The other drawback is that this system could only work on one sample at a time, therefore giving no measurement of concentration gradients which would be expected in as dynamic a system as the Miniclave chamber.

The fluorometric method was described by Lazrus *et al*, 1988. This is an automated system which initially strips formaldehyde vapour from an air sample into water at pH 2.0 by means of a glass coil. This aqueous solution of HCHO is then oxidised by Nicotinamide adenine dinucleotide (NAD^+) to form the reduced coenzyme NADH in the presence of formaldehyde dehydrogenase. This NADH produced can then be determined fluorometrically. This is a rapid system, and easily used to give proportional voltage/current output for reading on a chart recorder or to drive a positive feedback system. The disadvantage of the system is that it is probably too sensitive, giving a linear calibration curve only up to 100 ppb, which means a system of diluting a sample from the Miniclave would be required.

This method has advantages over the laser system as it would be cheaper, and its possible to have several sampling systems set up to measure concentration gradients. This method is also very specific as it is based on an enzyme reaction. The disadvantage is that this method still requires a sample to be removed from the chamber with its associated problems (condensation of formaldehyde, etc.).

The final method, that of using a piezoelectric crystal is by far the most promising and practical technique of the three. This method operates on the principle of a piezoelectric crystal vibrating at a particular frequency. If a molecule of a foreign substance attaches to the surface of the crystal, then that frequency is altered. This change in frequency can be related to the amount of compound that has attached by the Sauerbrey equation:

$$\Delta F = -\Delta M C$$

ΔF = frequency change in Hz,

C = constant (related to basic frequency, MHz),

ΔM = a factor to relate the mass adsorbed to a concentration (ppm)

If the surface of the crystal can be made specific for a particular molecule, then the concentration of that molecule can be accurately determined in a gas, even if a mixture of compounds is present. Guilbault (1983) achieved this by coating the crystal surface with formaldehyde dehydrogenase and NAD^+. The enzyme was isolated from *Pseudomonas putida*. This crystal then very specifically reacted with formaldehyde, even when in a mixture of gases. Guilbault demonstrated that the largest interferring

substance, acetaldehyde, gave only 1/50 the response of formaldehyde, and that methanol gave no reaction at all. These piezoelectric crystals are manufactured commercially, and are therefore relatively cheap, and the enzyme can be removed once its useful life is over, and the crystal recoated. The small sizes of these crystals would also allow them to be fitted inside the chamber, removing the need for samples to be taken, and allowing several points to be monitored simultaneously, hence measuring concentration gradients. The disadvantage of this system is that the formaldehyde dehydrogenase used by Guilbault would almost certainly degrade at temperatures in excess of 70°C as found in the Miniclave chamber. A more thermostable enzyme would need to be isolated or designed. Perhaps from a thermophile such as *Bacillus stearothermophilus*. Another disadvantage is that the enzyme on the crystal must have time to regenerate. For this to occur, the crystal must be exposed to the formaldehyde for less than one minute periods, then be flushed with clean air, or else the equation

$$\text{Enzyme + cofactors + formaldehyde} \underset{\text{clean air}}{\rightleftharpoons} \text{complex} \xrightarrow[1\text{ min}]{} \text{products}$$

will go completely to the right, ruining the crystals usefulness. This problem should easily be overcome however, by the use of solenoid valves to isolate the crystals, and air and vacuum lines to purge the crystal.

All of the methods described in this chapter have one major shortfall. This is that none of them in their present form can distinguish between monomeric and polymeric formaldehyde. It has

been suggested that it is the monomeric form of formaldehyde that is bactericidal, and it is therefore the proportion of this form of formaldehyde that is of chief concern. It may be that the enzyme reaction of the piezoelectric crystal or fluorometric method is that specific but if it is it has not been demonstrated. As enzyme reactions are very specific it is the most likely way of developing a specific monomer detector.

Assuming that the temperature and formaldehyde concentration can now be controlled with the systems discussed above, it was necessary to expose some spores of *B. stearothermophilus* NCIB 8224 to controlled LTSF conditions. To do this, it was necessary to choose a carrier system to form the basis of a test piece. Aluminium carriers were chosen and prepared as described in 4.3.2. Before these could be used routinely to expose spores to LTSF, a method of removing the spores from the carrier after exposure had to be selected. It was decided that sonication was an effective method of removal of spores and an experiment to determine the optimum time of sonication and if sonication has any detrimental effect on the spores was described in 4.3.2.3

From the results of these experiments shown in Table 4.4, it can be seen that after 10 min sonication the viable count of the solutions containing the sonicated carriers equals that of the controls. The five minute sonicated samples show a range of counts from 50-75% recovery of the total, and the fifteen minute sonication showed no increase over the 10 minute. This was confirmed by the t-test (Table 4.5) which demonstrates no significant difference between the mean of the 10 minute recovery and that of the untreated control. The t-test of the data from the 0 min control and the 15

min control demonstrates that at p=0.05, there is no significant difference, and therefore sonication has no effect on spore viability. Finally the analysis of variance on the number of colonies from spores from the carrier sonicated for 10 minutes demonstrated that there is no significant variation within or between and an overall coefficient of variation of 5% (Table 4.6).

Three conclusions can be drawn from these results. Firstly that the process of sonication had no effect on the viability of the spores (either activation or inactivation), a problem reported by Ordonez and Burgos, 1976. The second conclusion is that a sonication time of 10 minutes seems to be capable of recovering 100% of the spores from the carrier and that the sonication time beyond 10 minutes up to 15 minutes does not seem to be critical. This is different from the five minute figure reported by Chinyanganya (1989), determined by electron microscopy studies. It could be that preparation of the carriers for viewing in the electron microscope, such as passing through the air lock to vacuum, may have removed spores still on the carrier in this study, giving a lower estimate of the time required. Finally it has been demonstrated that this recovery technique is reproducible, and hence suitable for use in further studies.

The final study on the inactivation of spores of *B. stearothermophilus* NCIB 8224 sporulated on C-LTD medium is described in section 4.3.3. The data in Figure 4.12 illustrates the reproducibility of the survivor curve, which is confirmed by the t-test analysis used to compare the slopes and intercepts of these lines (Table 4.7). The linear nature of these survivor curves is a distinct improvement on the data obtained by Chinyanganya (1989),

using the single formalin injection system. The biphasic curves obtained in that study were almost certainly due to loss of formaldehyde from the chamber as discussed previously.

The final conclusion that can be drawn from the work reported in this chapter is that the objective of the modification of the Miniclave 80 was to produce an experimental test apparatus to expose spores of a potential BI organism to controlled LTSF conditions. The results reported here demonstrate that this objective was attained.

The MBTH method used in this study was successful in that it was reproducible if not altogether accurate. This means that on the basis of this measuring system, reproducible experimental conditions could be set up to test bacterial spores, and as shown by results in chapter 5, even after concentration and temperature settings were altered, setting them back at their original settings resulted in the same rate of inactivation of bacterial spores of NCIB 8224, thereby demonstrating the methods reliability. This means that it is a valid method of setting experimental conditions of formaldehyde concentrations as long as its weaknesses are recognised.

CHAPTER 5

CHARACTERISATION OF RESISTANCE OF SPORES OF BACILLUS STEAROTHERMOPHILUS NCIB 8224 TO INACTIVATION BY LTSF

5.1 INTRODUCTION

Chapter 3 reported on the initial screening of spores of a number of *B. stearothermophilus* strains for suitability as a biological indicator for LTSF. This was primarily based on the resistance of the spores to aqueous formaldehyde at 70°C. The construction of the experimental apparatus described in Chapter 4 made it possible to measure formaldehyde concentrations in the chamber. The reproducibility of survivor curves constructed with data obtained using the modified Miniclave 80 was demonstrated in section 4.3.3.

This chapter reports on work carried out to determine the resistance of spores of *B. stearothermophilus* NCIB 8224 produced on C-limited medium to various LTSF conditions.

5.2 EXPERIMENTAL

5.2.1 Effect of Temperature on the Inactivation of Spores of *B. stearothermophilus* NCIB 8224 by LTSF.

Test pieces were prepared by drying spores of *Bacillus stearothermophilus* NCIB 8224 produced on C-limited medium on to aluminium cup carriers as described in section 4.3.2.1. These were stored in the dark at 4°C for a maximum of 24 hours prior to use. The Cal 9000 temperature controller was set to the operating temperature, and the controllers for the jacket and the door set 2°C below this temperature. The first syringe (initial injection) volume and the second syringe ("top-up" injection) interval timers were adjusted to give the required concentration of formaldehyde in the chamber as described in section 4.3.1.2. As the Miniclave 80 cycle characteristics alter with the operating temperature, the settings for the first and second syringes needed to be found by

experimentation for each operating temperature. The method for assaying gaseous formaldehyde described in section 4.3.1.2 was used to monitor concentrations both in the preliminary setting up and during inactivation experiments. A concentration of 12 $\mu g ml^{-1}$ concentration of formaldehyde was chosen for use in the experiment to discover the effect of temperature. This was chosen as this concentration had already been used in the previous chapter and was found to give a D-value which allowed practical sampling times for experimentation, using the test organism at 73°C. The test pieces were loaded into the sample holders and inserted into the secondary chambers on the door using the sequence illustrated in Figure 4.8 (A-E). The Miniclave 80 was allowed to run until the initial vacuum pulses finished. The samples were not introduced into the chamber until a further three minutes after the primary injection. This time was allowed to let the conditions in the chamber stabilize. The samples were then introduced into the main chamber, after a vacuum was drawn in the secondary chamber for 60 seconds. Samples were withdrawn at predetermined intervals. They were allowed to equilibriate up to atmospheric pressure in the secondary chamber for five seconds by opening the air line, before finally removing the sample holder. The test pieces were then aseptically transferred into thin walled screw-top bottles containing 10 ml of 1% glycine solution to inactivate any formaldehyde carried over on the test pieces. The vessels were then suspended in an ultrasonic bath and sonicated at 51 kHz for 10 minutes (section 4.3.2.3) and the surviving organisms recovered on Nutrient Agar using the spread plate technique described in section 2.3.1.3. The temperatures studied were 63°C, 68°C, 73°C, 78°C and 83°C. These were chosen to cover a range of ±10°C of the operating temperature recommended for LTSF

sterilization in the U.K. (73°C). They were also chosen to reduce the chances of overlap at different settings, as the temperature control on the Miniclave 80 was only accurate to approximately ±1°C. At least three replicates of an inactivation experiment were carried out at each temperature. The data were then pooled, and linear regression analysis carried out to plot the best fit line and to calculate the correlation coefficients for a straight line model. The survivor curves constructed from these data with regression lines and Standard Error bars (calculated as described in Appendix III) are shown in Figures 5.1 - 5.5 and the intercepts, slopes and correlation coefficients obtained by regression analysis for each curve are recorded in Table 5.1

5.2.1.1 Treatment of Results

In work published by previous authors, the relationship between temperature and inactivation of spores has been plotted as a "pseudo-Arrhenius" plot (Hoxey, 1984; Hurrell, 1988; Chinyanganya, 1989). This was a technique where T_3 values were used instead of k values due to the problems of calculating k values for non-linear survival curves.

The data in Table 5.1 indicate that none of the survivor curves obtained in these experiments (figs. 5.1-5.5) deviates significantly from a linear relationship (R^2 > 95% in all but the 63°C test), and the error bars demonstrate their reproducibility. The rate constant (k) can therefore be used as a valid measure of the efficiency of the sterilant (as opposed to T_3 values). The rate constant (k) is related to the number of viable spores as follows:

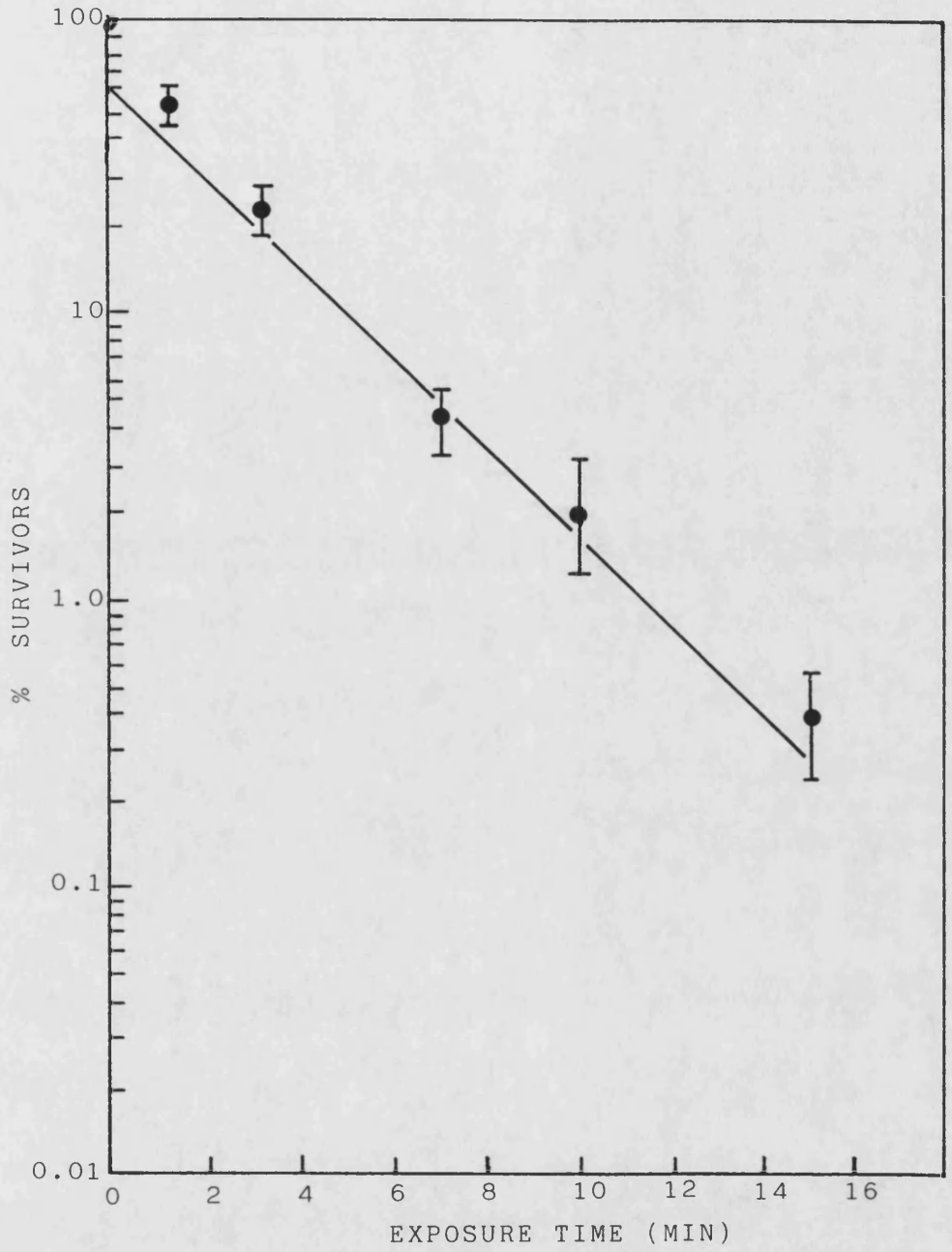


Figure 5.1 Survivor Curve For Spores of B. stearothermophilus NCIB 8224 Produced on C-Limited Medium when Exposed to LTSF at 63°C and 12 ug/ml Formaldehyde Concentration.

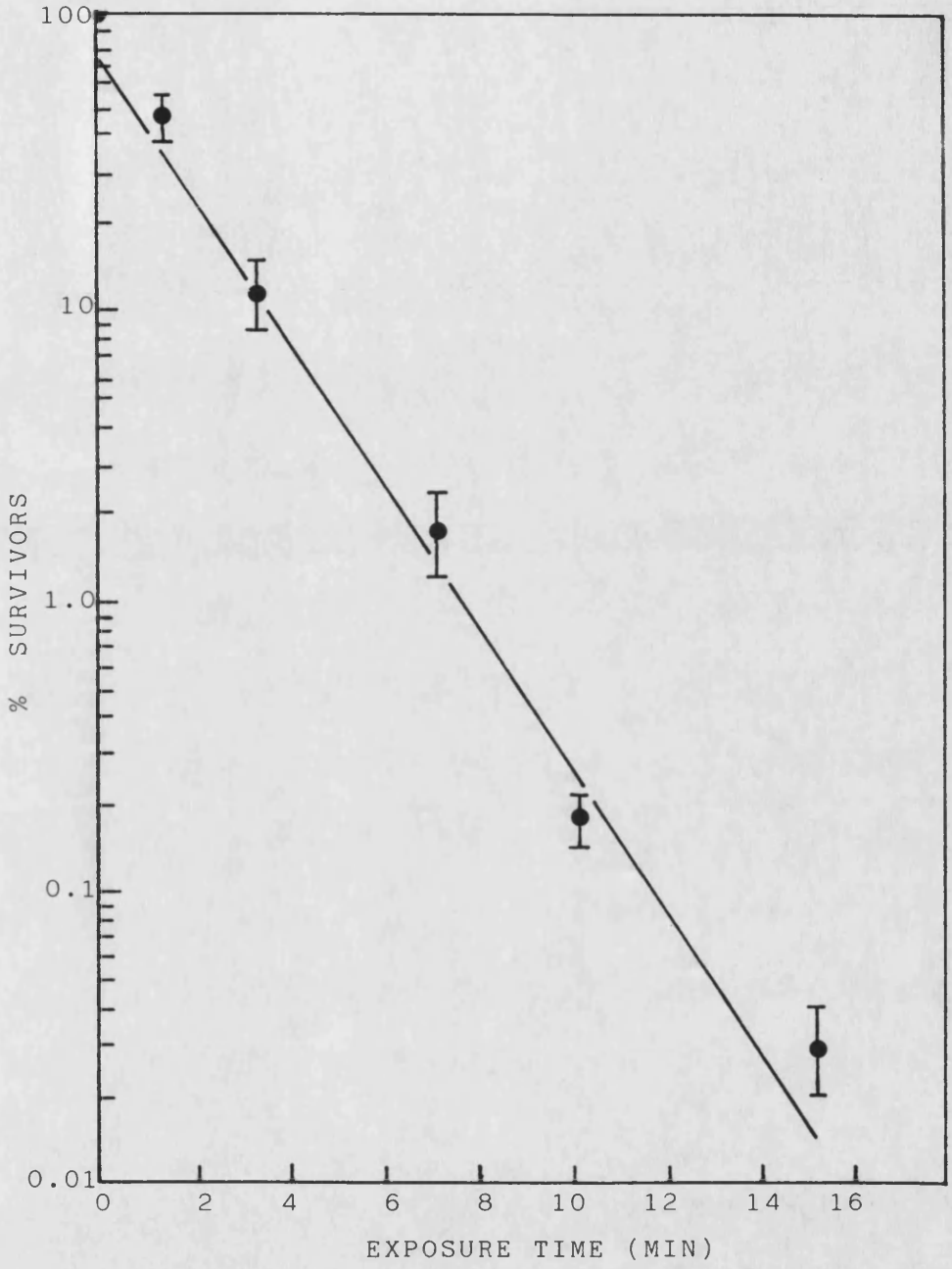


Figure 5.2 Survivor Curve For Spores of B. stearothermophilus NCIB 8224 Produced on C-Limited Medium when Exposed to LTSF at 68°C with 12 ug/ml Formaldehyde Concentration.

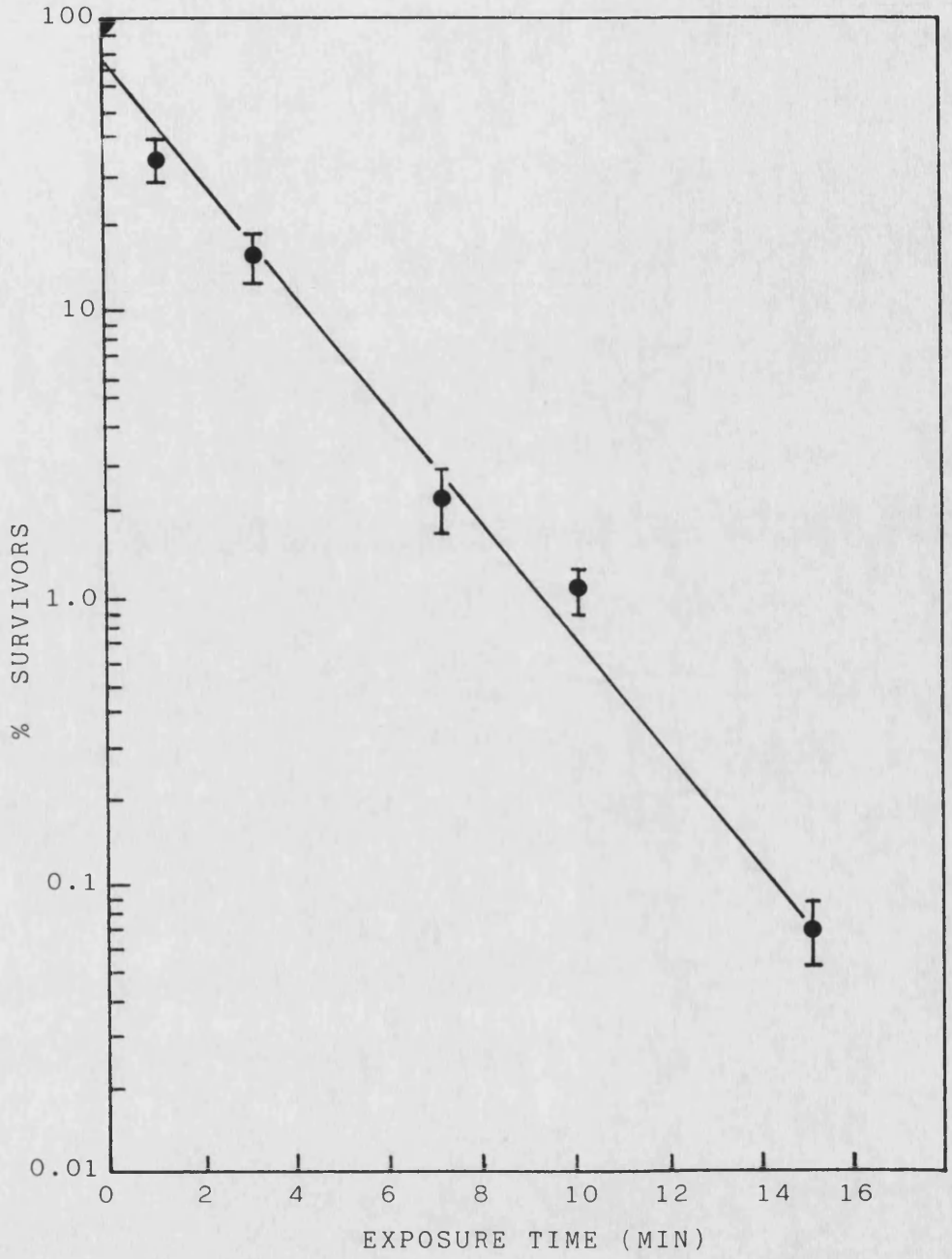


Figure 5.3 Survivor Curve for Spores of B. stearothermophilus NCIB 8224 Produced on C-Limited Medium when Exposed to LTSF at 73°C with 12 ug/ml Formaldehyde Concentration.

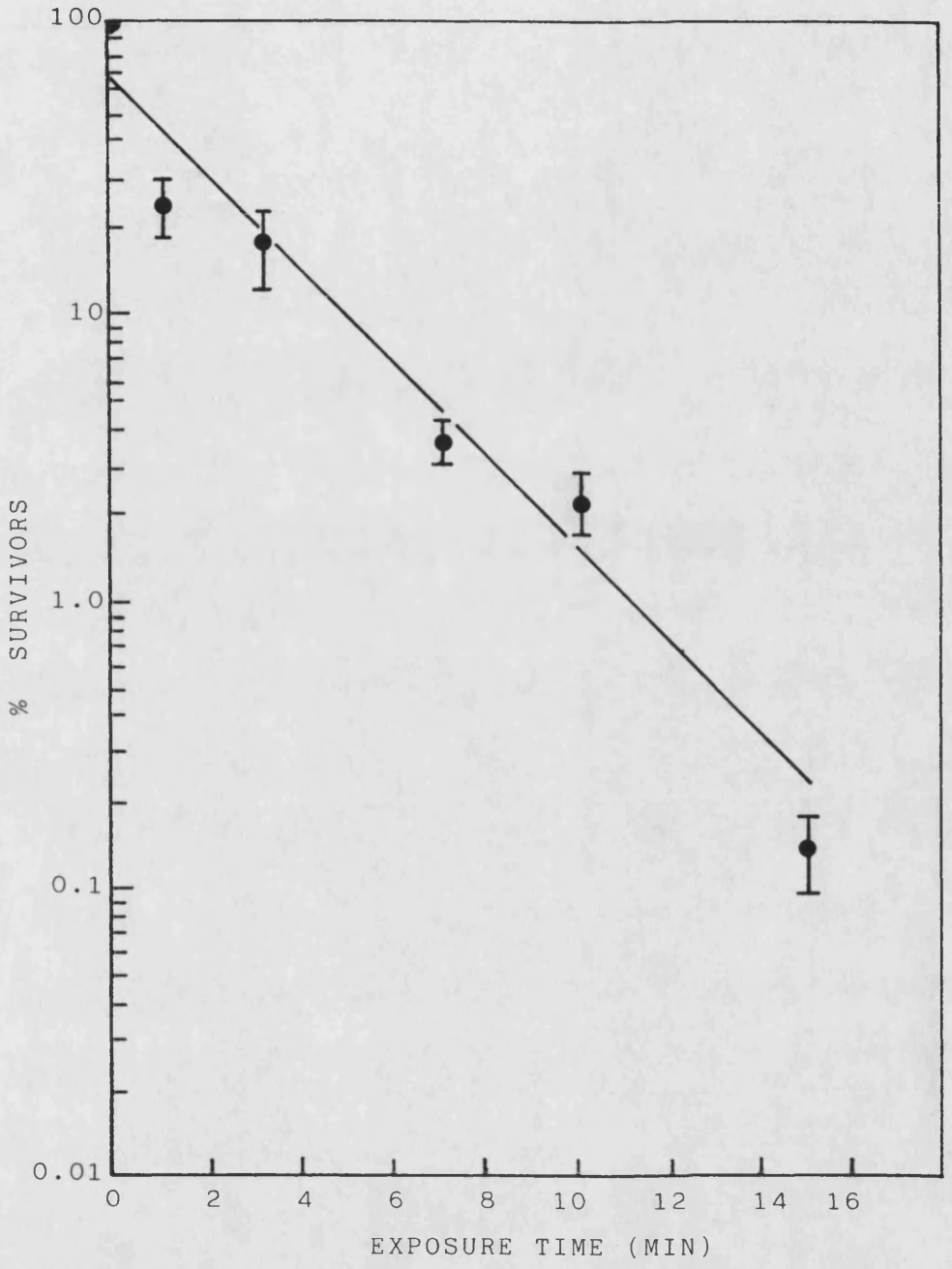


Figure 5.4 Survivor Curve For Spores of B. stearothermophilus NCIB 8224 Produced on C-Limited Medium when Exposed to LTSF at 78°C with 12 ug/ml Formaldehyde Concentration.

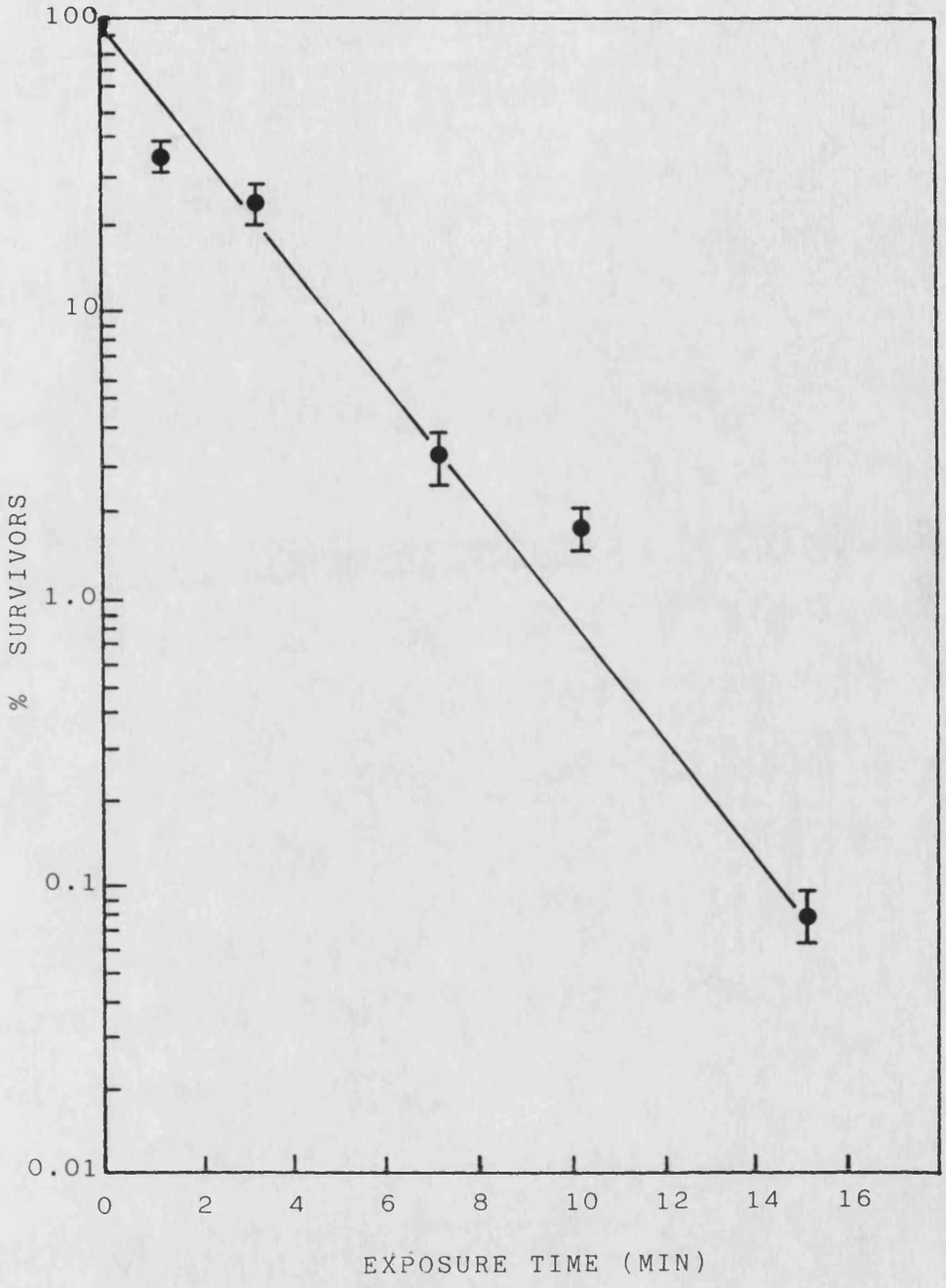


Figure 5.5 Survivor Curve For Spores of B. stearothermophilus NCIB 8224 Produced on C-Limited Medium when Exposed to LTSF at 83°C with 12 ug/ml Formaldehyde Concentration.

Table 5.1 Intercepts and Rate Constants (k) Obtained by Regression Analysis of Survivor Curves of Spores of *B. stearothermophilus* NCIB 8224 Produced on C-LTD Medium when Exposed to LTSF at a Formaldehyde Concentration of 12 µg/ml Over a Range of Temperatures

Absolute Temperature (K)	Log % Intercept	Slope (min^{-1})	k (min^{-1})
336	1.77	-0.1599	0.368
341	1.86	-0.2476	0.570
346	1.87	-0.2073	0.477
351	1.82	-0.1643	0.378
356	1.99	-0.2095	0.483

$$k = 1/t \ln \frac{N_0}{N}$$

Where k = rate constant (time $^{-1}$)
N_0 = Initial number of viable organisms
N = Final number of viable organisms
t = time for viable count to fall from N to N_0 (time)

The rate constant can be obtained graphically as the slope of a survivor curve plotted as percentage survivors on a Log scale against exposure time on a linear scale. The k values for the curves illustrated in Figures 5.1-5.5 are also given in Table 5.1

As the survivor curves follow first order kinetics, and hence k is a valid measure of the inactivation rate, it is possible to relate the temperature of inactivation to the rate constant by the Arrhenius equation :

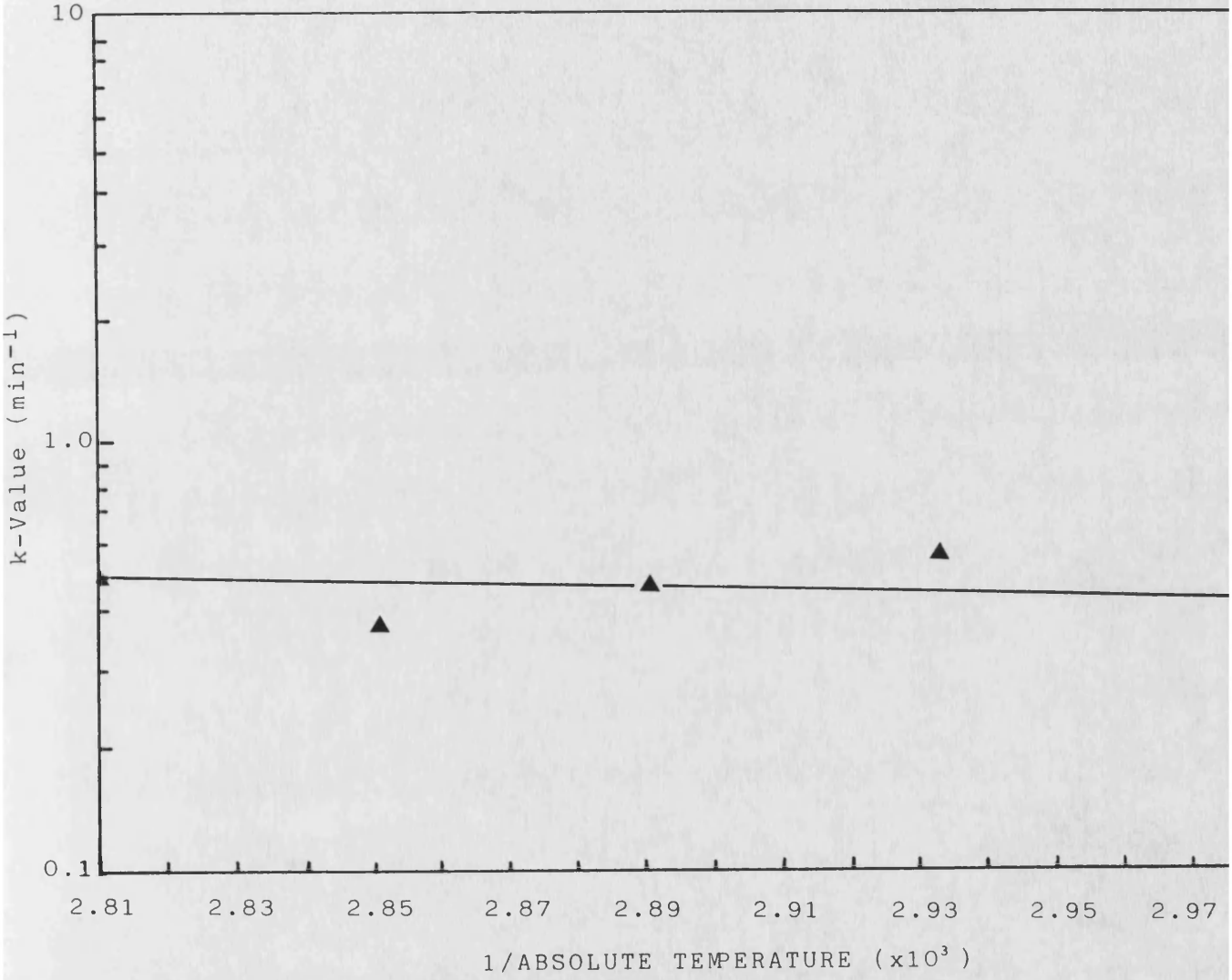
$$k = Ae^{-Ea/RT}$$

Where k = Rate constant (time^{-1})
A = Frequency factor (time $^{-1}$)
Ea = Activation energy of bacterial death
R = Universal gas constant
T = Absolute temperature (K)

Taking Logs of both sides of this equation it becomes :

$$\text{Log } k = \text{Log } A - Ea/2.303\ RT$$

An Arrhenius plot is obtained by plotting the rate constant on a log scale against the reciprocal of the corresponding Absolute temperature on a linear scale. On such a plot, the slope of the line is equal to Ea/2.303R. The result of plotting this for the data obtained here is shown in Figure 5.6. This graph shows that there is



igure 5.6 Plot of Inactivation Rate Constant (k) Against Reciprocal of the Absolute Temperature (Arrhenius Plot) for Spores of B. stearothermophilus NCIB 8224 Produced on C-limited Medium after Exposure to LTSF at 12 ug/ml Formaldehyde Concentration Over a Range of Temperatures.

no significant difference in the inactivation rate at any of the temperatures used. There is a variation apparent, but this is probably due to slight cycle variations which are unavoidable. There appears to be no trend, and any difference can probably be accounted for by slight variations in the range of formaldehyde concentrations in different cycles. This result will be discussed in detail in section 5.3. The standard error bars indicate that the data used to construct the survivor curves illustrated in Figures 5.1-5.5, and in particlar 5.1, exhibit variation around the mean point which is not greatly different from that obtained in aqueous inactivation experiments, indicating the reproducibility of data obtained using the modified Miniclave 80.

5.2.2 Effect of formaldehyde concentration on the inactivation of *B. stearothermophilus* NCIB 8224 spores produced on C-limited medium

A formaldehyde concentration of 8 μgml^{-1} is commonly used in LTSF sterilization cycles in the U.K. In previous studies, formaldehyde concentrations between 6 μgml^{-1} and 27 μgml^{-1} have been studied (Hoxey, 1984). In this investigation formaldehyde concentrations in the range 2.9 μgml^{-1} to 17.2 μgml^{-1} were selected, partly to facilitate comparison of the experimental data with those of Hoxey and partly due to limitations of the Miniclave 80.

The controls of the Miniclave 80 were adjusted to maintain a temperature of 73°C ± 1°C. Test pieces of *B. stearothermophilus* NCIB 8224 spores produced on C-limited medium were produced as described in section 4.3.2.1. The syringes were adjusted, as in section 5.2.1, to give the required concentration. The gas in the chamber was assayed using the method outlined in 4.3.1.2 to determine the concentration of formaldehyde in the chamber both during the

preliminary setting up and throughout the inactivation experiment. Samples were introduced into the chamber as detailed in section 5.2.1, and removed at predetermined intervals. The number of survivors was ascertained as in section 5.2.1. A minimum of three replicate determinations were carried out at each formaldehyde concentration, the data pooled, and mean survivor curves constructed (Figures 5.7 - 5.10). The curves were analysed by linear regression analysis and the data obtained (including the intercepts, slopes and correlation coefficients) are given in Table 5.2. As these survivor curves are all linear, as shown by their high correlation coefficients, it is valid to use inactivation rate constants (k) to express the lethality of the process at the different concentrations. These k values, (calculated from $-k = 2.303 \times$ slope), are also given in Table 5.2. One way of demonstrating the effect of formaldehyde concentration on the lethal effect of the process is to plot the inactivation rate constants (k) on a linear scale against the formaldehyde concentration on a linear scale, and this is shown in Figure 5.11. This plot demonstrates that there is an increase in the rate of inactivation of spores with increasing concentration of formaldehyde. This relationship is linear up to a concentration of approximately 12 $\mu g ml^{-1}$ at which point there is no further increase in the rate of inactivation. The implications of this will be discussed in detail in section 5.3.

As the data illustrated in Figure 5.11 shows a linear relationship for formaldehyde concentrations up to 12 $\mu g ml^{-1}$, another method of demonstrating the effect of formaldehyde concentration on spore inactivation, the concentration exponent (n), can be used. The concentration exponent is defined as the slope of the plot of death time (e.g. D-value) on a log scale against the concentration of

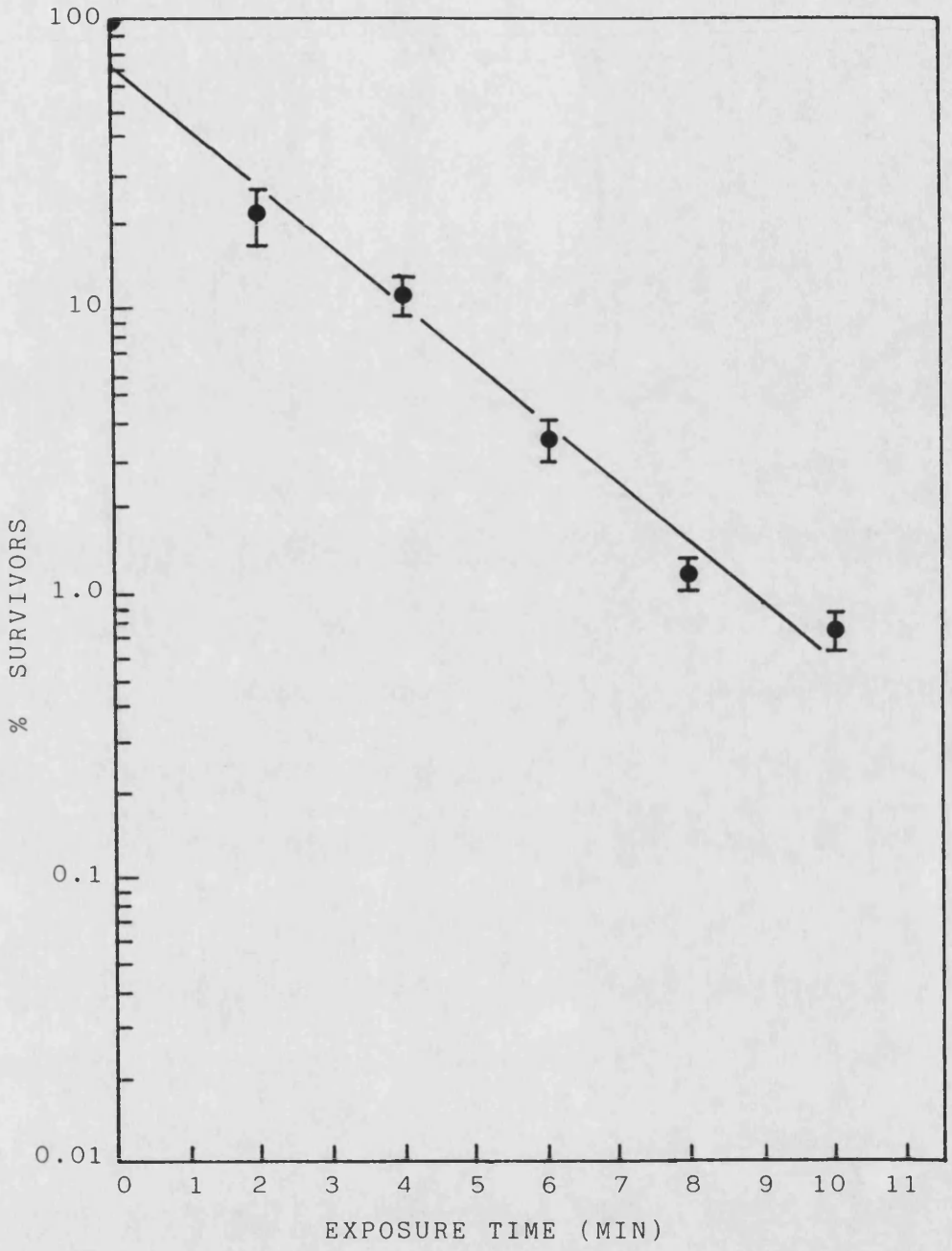


Figure 5.7 Survivor Curve for Spores of B. stearothermophilus NCIB 8224 Produced on C-limited Medium When Exposed to LTSF at 73°C and 17.2 ug/ml Formaldehyde Concentration.

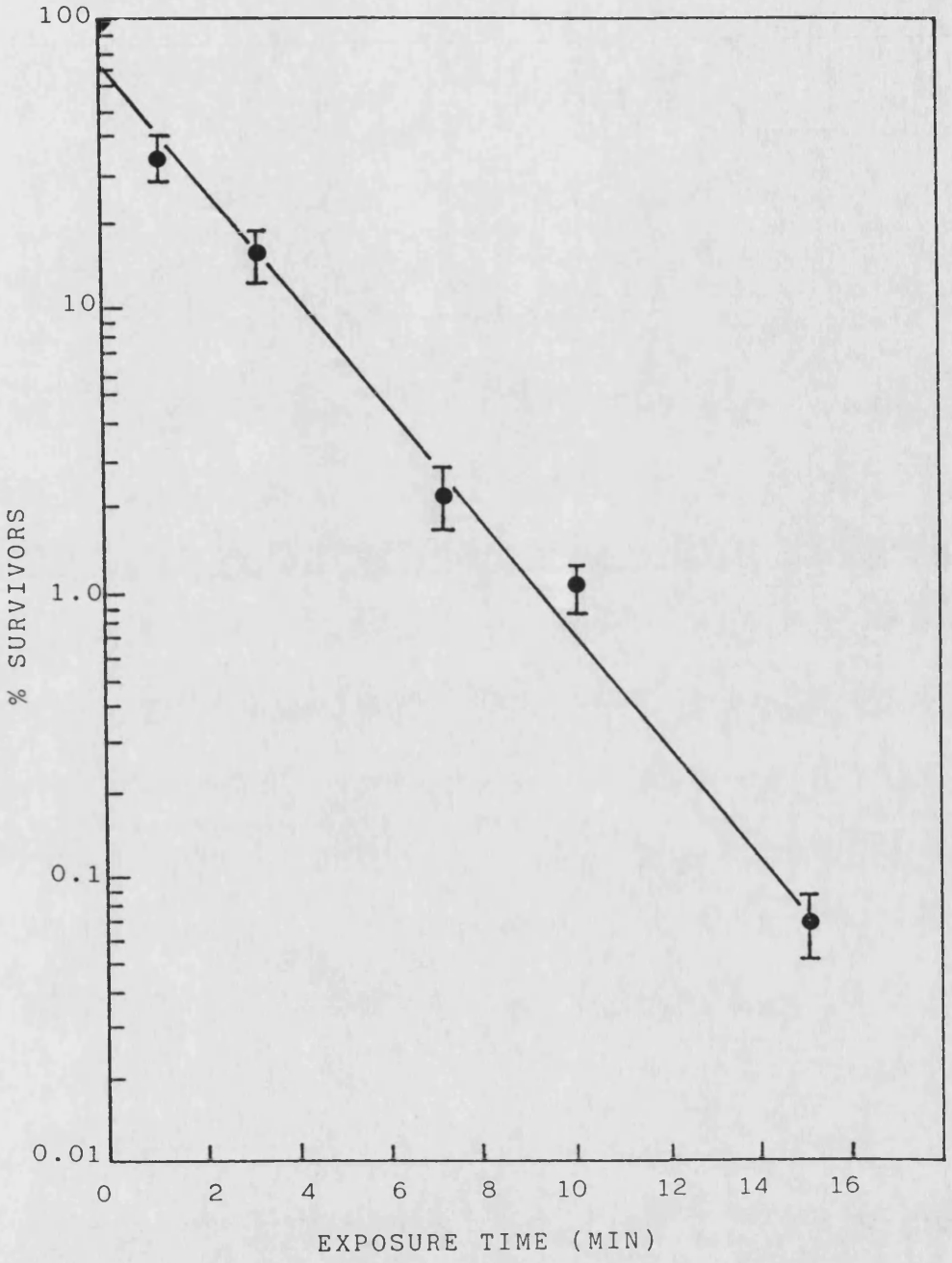


Figure 5.8 Survivor Curve for Spores of B. stearothermophilus NCIB 8224 Produced on C-limited Medium When Exposed to LTSF at 73°C With 12 ug/ml Formaldehyde Concentration

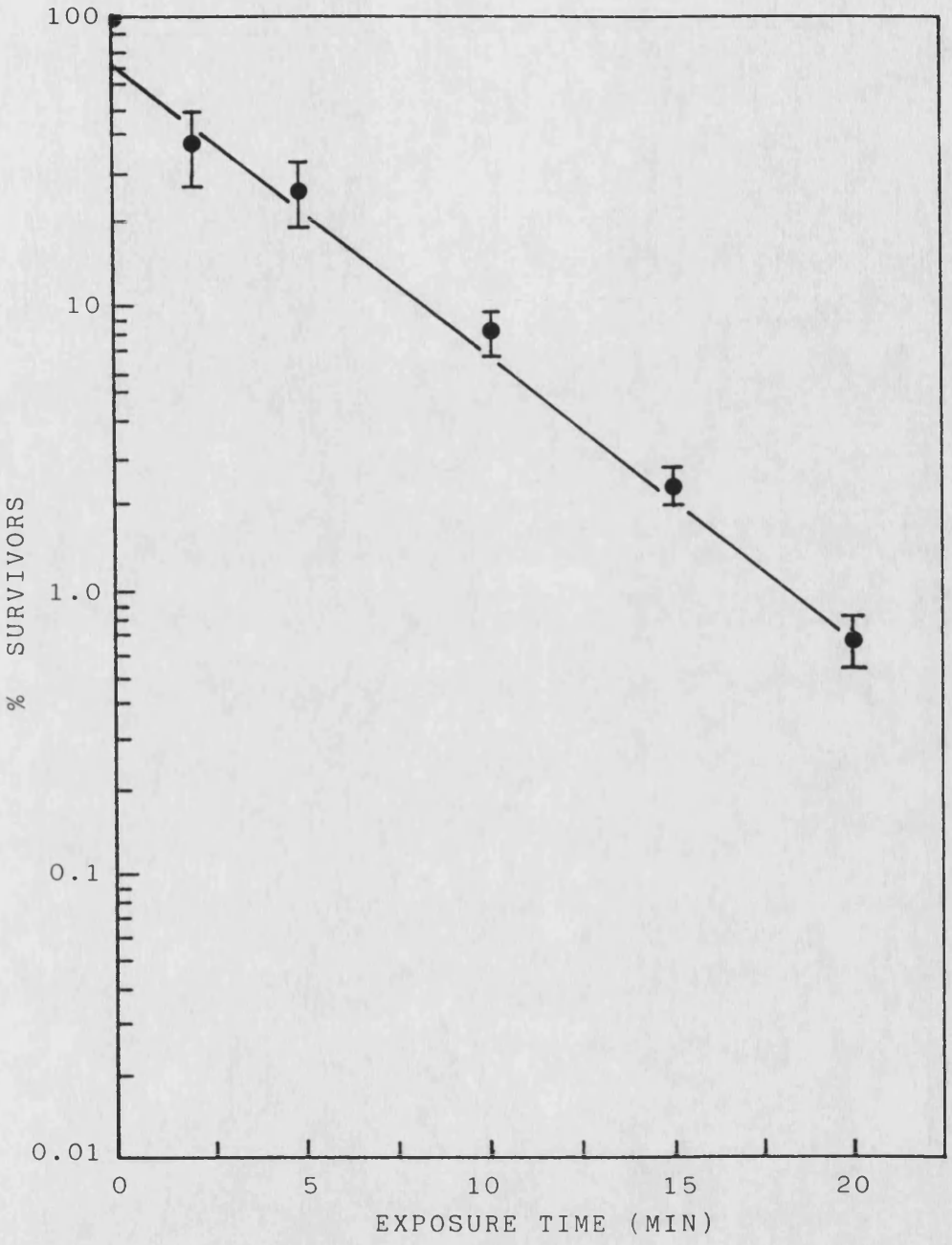


Figure 5.9 Survivor Curve for Spores of B. stearothermophilus NCIB 8224 Produced on C-limited Medium When Exposed to LTSF at 73°C and 6.7% Formaldehyde Concentration.

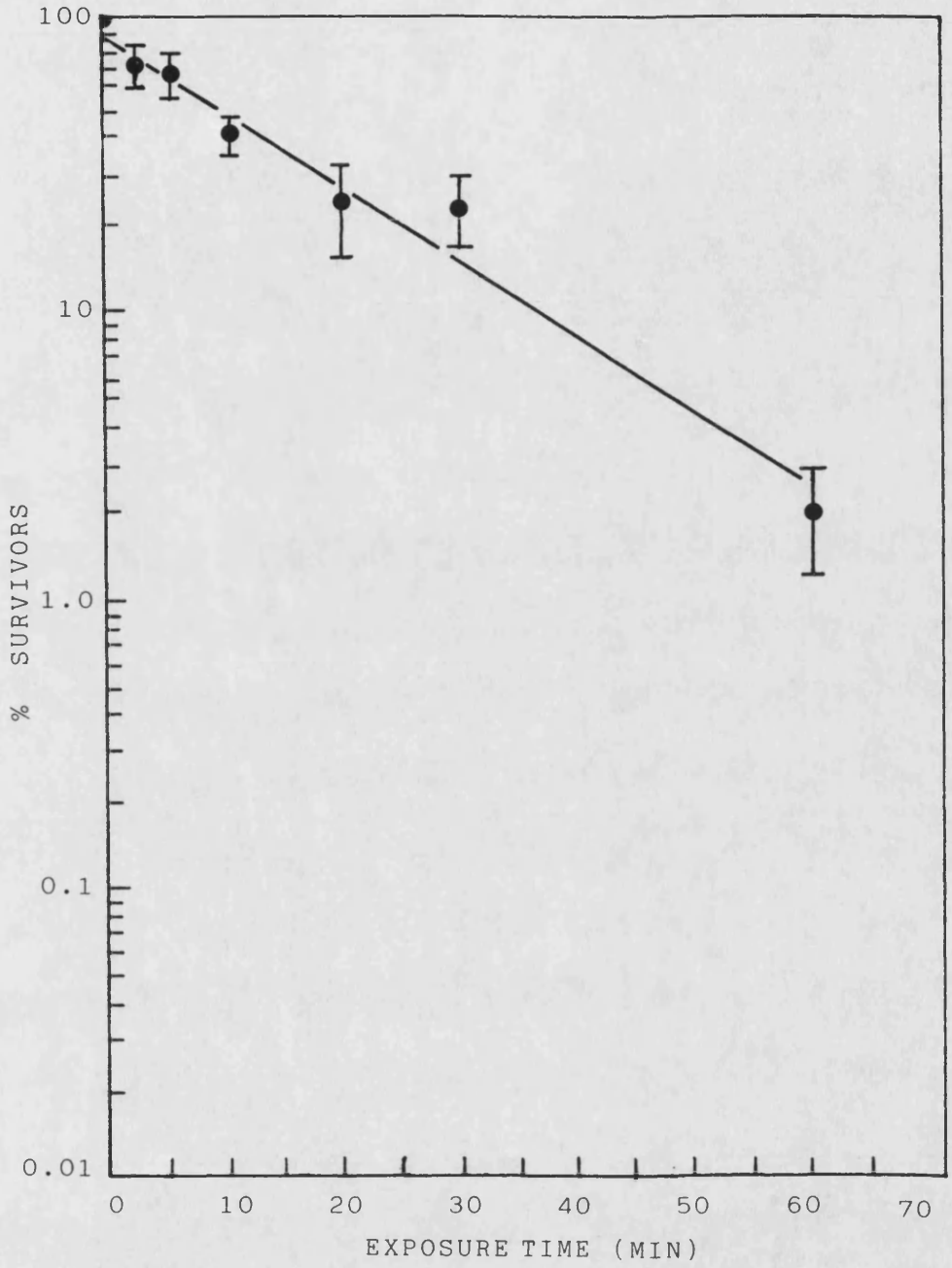


Figure 5.10 Survivor Curve for B.stearothermophilus NCIB 8224 Produced on C-limited Medium When Exposed to LTSF at 73°C and 2.9 ug/ml Formaldehyde Concentration.

Table 5.2 Intercepts, Slopes, Correlation Coefficients (R^2) and Inactivation Constants (k) obtained by Regression Analysis of Survivor curves of Spores of *B. stearothermophilus* NCIB 8224 Produced on C-limited Medium When Exposed to LTSF at 73°C at a Range of Formaldehyde Concentrations

Concentration (µg/ml)	Log % Intercept	Slope	R^2 (%)	k (Min^{-1})
17.2	1.86	-0.20834	97.5	0.48
12.0	1.87	-0.20728	96.7	0.48
6.7	1.85	-0.10080	91.5	0.23
2.9	1.94	-0.02495	97.1	0.058

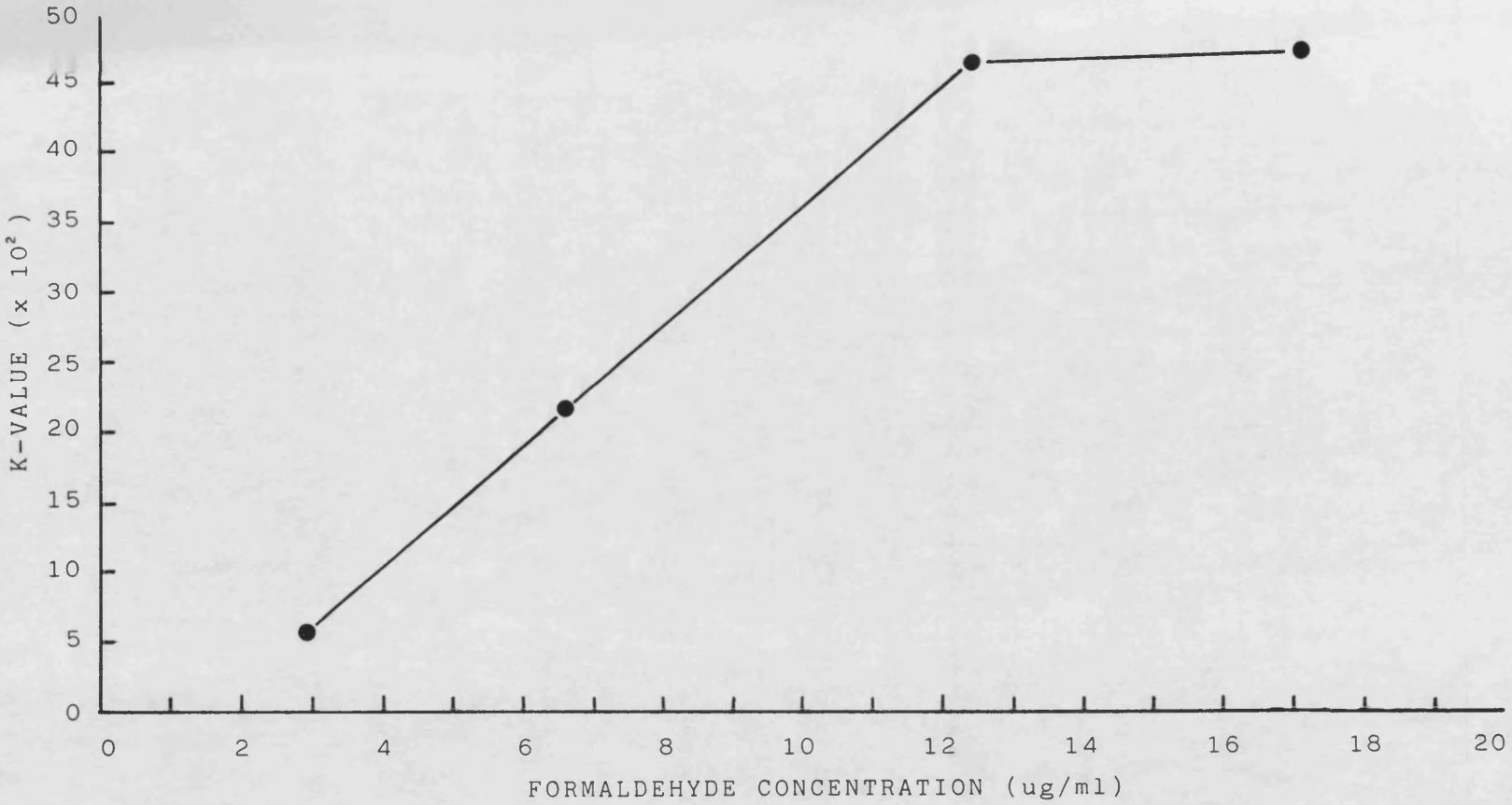


Figure 5.11 Plot of Inactivation Rate Constants (k) Against Formaldehyde Concentration for Spores of B. stearothermophilus NCIB 8224 Produced on C-limited Medium after Exposure to LTSF at 73°C over a Range of Formaldehyde Concentrations.

inactivating chemical on a Log scale. The D-value can be calculated from the relationship : D = 2.303/k.

The plot of Log 2.303/k against Log concentration, for formaldehyde concentrations between 2.9 and 12 μgml^{-1}, is illustrated in Figure 5.12. Table 5.3 shows the regression data for this line, from which it can be seen that there is a positive correlation between concentration and inactivation rate (R^2 = 99.6%). The value for the concentration exponent is approximately 1.4. The significance of this value will be discussed in section 5.3.

Table 5.3 Regression data for Plot of Log Formaldehyde Concentration against Log 2.303/k for Survivor Curves of Spores of B. stearothermophilus NCIB 8224 Produced on C-limited Medium When Exposed to LTSF at 73°C Over a Range of Formaldehyde Concentrations.

Data

Log Concentration	Log 2.303/k
0.46	1.60
0.83	1.00
1.08	0.68

Regression Analysis

Parameter	Estimate	Standard error
Intercept	2.27	0.078
Slope	-1.41	0.094

Correlation Coefficient = 99.60%

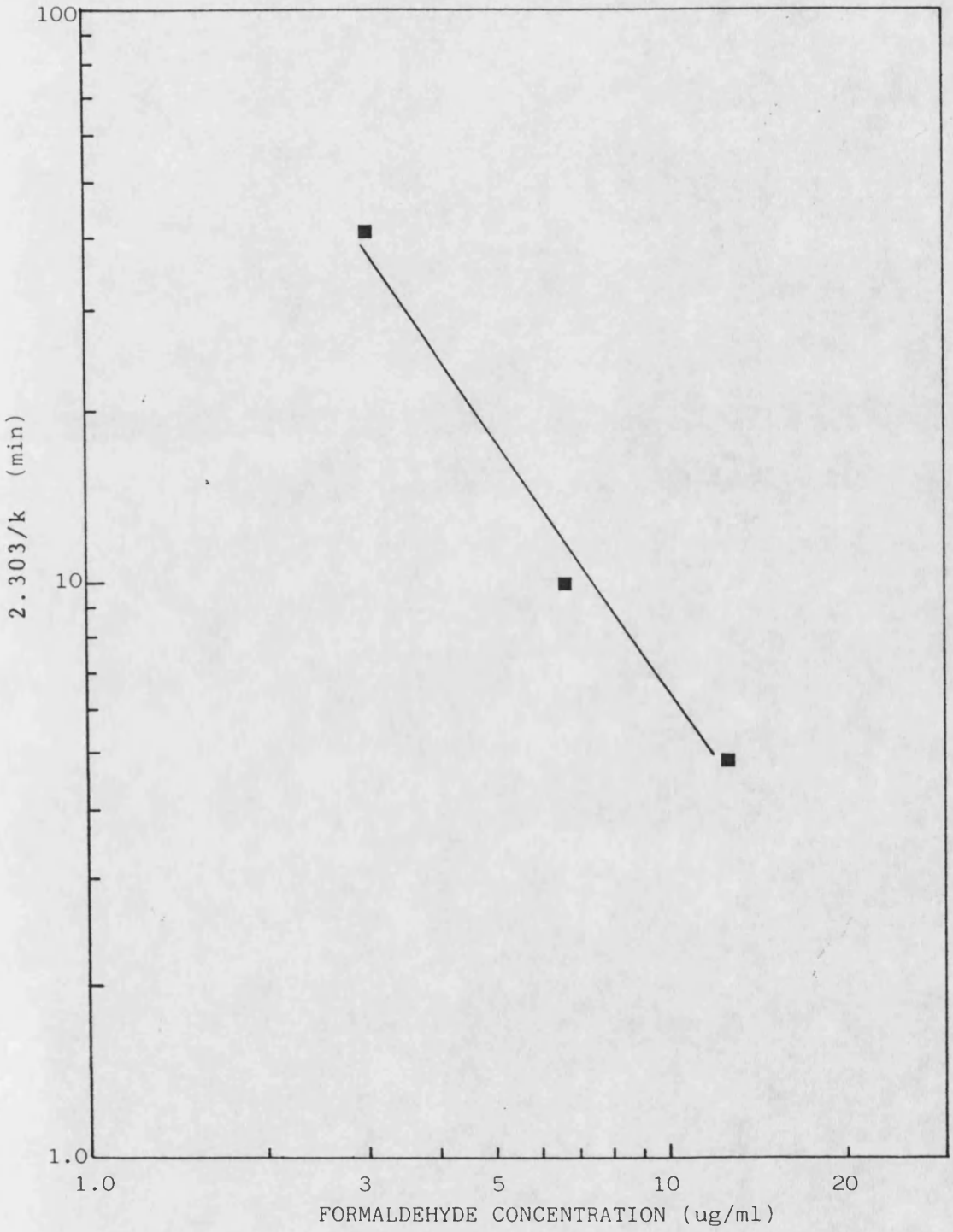


Figure 5.12 Plot of Log $\frac{2.303}{k}$ against Log Formaldehyde Concentration for the Inactivation of Spores of B. stearothermophilus NCIB 8224 Produced on C-limited Medium When Exposed to LTSF with 12 ug/ml at 73°C

5.3 DISCUSSION

The experiments reported in this chapter have demonstrated that the development of a semi-automatic test apparatus, for LTSF inactivation studies is possible. More importantly, it has shown that such an apparatus can be used to produce reproducible survival curves of a range of temperatures and formaldehyde concentrations. The range of temperatures studied was 63°C to 83°C at concentrations ranging from approximately 3 µg/ml to 17 µg/ml. This concentration range covers the more regular ones used such as the 15 µg/ml used in routine LTSF cycles (CSC report, 1986), though concentrations as high as 100 µg/ml have been reported (Alder, 1968; Russell, 1982).

Little work has been published with quantitative survival data for spores inactivated under LTSF conditions. Pickerill (1975) demonstrated that LTSF had very high sporocidal properties. This was shown by proving that no surviving organisms could be recovered after exposing spore strips of *Clostridium sporogenes, B. globigii, B. pumilis* and *B. stearothermophilus* to LTSF treatments. The cycle parameters used in that study were a formalizing stage, followed by a 20 minute holding period at approximately 50 µg/ml. These were carried out over a range of temperatures between 65°C and 80°C. Alder (1968) exposed 4.8×10^5 spores of *B. subtilis*, and 9.0×10^5 spores of *B. stearothermophilus* to 100 µg/ml gaseous formaldehyde at temperatures of 80°C and 90°C, for up to 40 minutes. He reported that no survivors could be recovered after this time.

It was not until later that quantitative survival data were obtained for organisms exposed to LTSF. Hoxey (1984) described the construction of an apparatus with six vessels, within which it was possible to set up various LTSF conditions. It was possible with

this apparatus to obtain samples taken at various time periods during the LTSF cycle. The data he published demonstrated survival curves exhibiting reductions in surviving spores of up to three log cycles. The most resistant of the organisms he investigated, the number of survivors of spores of *B. stearothermophilus* NCIB 8224 sporulated in liquid SSMAVIT, dropped three log cycles in 3.5 minutes, with 27 µg/ml formaldehyde at 80°C demonstrating the high lethality of LTSF. However, this result, and all of the others reported were all non-linear in inactivation kinetics. The apparatus described in his work was also very large, complex and would be difficult to use and maintain for long investigations. Chinyanganya (1989) reported on the modification of the Miniclave 80, a commercial LTSF sterilizer, allowing variable cycles to be produced. Due to the limitations of the machine at that time, no direct measurement of formaldehyde concentrations could be made. The results of these investigations demonstrated biphasic survival curves for spores of NCIB 8224 sporulated on C-limited medium. One part of the curve exhibited linear kinetics, but rapidly tailed off after only one log cycle reduction in survivors. This tailing was almost certainly due to the loss of formaldehyde demonstrated in Figure 4.11, for which no compensation could be made at this stage of development of the Miniclave 80.

The results illustrated in Figure 4.12 demonstrate that with the full modifications described in Chapter 4 it was possible to hold a steady concentration of formaldehyde in the chamber. A steady formaldehyde concentration allowed inactivation experiments to be carried out for periods causing three log cycle drops in survivors of *B. stearothermophilus* NCIB 8224 spores. The limit of three log cycles was not due to limitations of the apparatus, but the test

pieces. As only 1 x 10^6 spores were loaded on a test piece, and the first dilution was into 10 ml of 1% glycine, this limited the number of cycles reduction that could be reliably recorded. A possible way around this would be to filter the first dilution by the method described in section 3.3.4.2. All of the survival curves detailed in figures 5.1-5.5 exhibit logarithmic (1st order) inactivation kinetics. This appears to uphold the theory that the biphasic survivor curves of Chinyanganya (with the same organism sporulated on the same medium) were artifacts due to the loss of formaldehyde from the Miniclave 80 chamber during inactivation studies, and not characteristics of the spores. Table 5.1 shows a summary of the characteristics of the survival curves produced over the range of temperature. All but the 63°C inactivation experiment show correlations (R^2) of >95% to a linear model. The 63°C has an R^2 value of 85% which demonstrates a greater variation from linearity at this temperature. This value reflects the fact that the 63°C cycle exhibited the highest fluctuations in pressure and temperature of all the temperatures tried. This is most probably due to overshoot caused by heating the chamber with steam at 110°C. Very little steam at this temperature is required to maintain 63°C, and the time required to open and shut the solenoid valves allowed the temperature to overshoot slightly (±1.5°C). This could be remedied by restricting the flow of steam to a much slower rate for cycles at this low a temperature.

From the k values of the survival curves (listed in Table 5.1), it was possible to construct an Arrhenius plot. This is the normal way of demonstrating the effect of temperature on reactions, (though S values based on 2 log cycle reductions in survivors have been used in the case of non-linear data (Hoxey, 1984)). The Arrhenius plot is

illustrated in Figure 5.6. This shows an almost level plot, indicating that increases in temperature have little effect on the sporicidal activity of LTSF. This result agrees with data obtained by Hoxey (1984) and Chinyanganya (1989) over a similar range of temperatures. A likely explanation for this is that the energy available in the low temperature steam increases only a little over the range of 63°C to 83°C. Table 5.4 illustrates the energy available in steam between temperatures of 60.06°C to 83.72°C.

Over this range, the sensible heat of the steam rises approximately 100 kJ/kg. At the same time however, the latent heat energy drops 59 kJ/kg. This leaves a net rise in total energy of only 41 kJ/kg, a 1.57% rise in the available energy. This means that although the temperature of the steam has risen 20°C the energy available to inactivate the spore has risen very little, and hence has little effect on the rate of inactivation.

Table 5.1 lists the intercept on the Y-axis of figures 5.1-5.5, obtained from the linear regression equation. In all cases, this figure is less than 100%, but is not consistent, showing an increase in intercept with increase in temperature. One explanation for this could be that some of the spores are being physically lost off the test pieces. The pulling of an initial vacuum in the secondary chamber, prior to opening the valve to the main chamber, is carried out to prevent this, as similar problems were reported by Hoxey (1984). The time required to draw a vacuum sufficient to prevent physical loss was determined by Chinyanganya (1989). To try to determine if this vacuum stage was insufficient, the difference in atmospheric pressure to chamber operating pressure, at various temperatures, was plotted against intercept on the Y-axis. This plot

is illustrated in Figure 5.13. It appears that there is a linear relationship between differential pressure and the estimated zero time count. An increase in differential pressure causes a lower estimate of the intercept. This could be due to there being more turbulence on opening the main valve at higher differential pressures, hence losing more spores. To demonstrate this conclusively, more investigation would be required, such as direct counting of spores before and after opening the valve, using equipment such as a Coulter Counter.

The final part of this investigation was designed to determine the effect of formaldehyde concentration on inactivation of spores at 73°C ±1.0°C. The concentrations used were approximately 17, 12, 6 and 3 μg/ml. Unlike temperature, formaldehyde concentration had a pronounced effect on inactivation rates, giving a range of k values from 0.057 at the lowest concentration (Figure 5.10) to 0.479 at the highest (Figure 5.7). From the data for the four concentrations used, it was possible to plot formaldehyde concentration against k to demonstrate the effect of formaldehyde concentration on the rate of inactivation of spores. This plot, shown in Figure 5.11 indicates that the rate of inactivation increases with increasing formaldehyde concentration up to a concentration of approximately 12 $\mu g ml^{-1}$, at which point no further increase is observed. This would seem to suggest that some sort of saturation point is reached at that concentration. This could be due to all the alkylation sites being filled. This has important implications for possible commercial cycle parameters as it indicates that approximately 12 $\mu g ml^{-1}$ may be the maximum useful concentration of formaldehyde. This value is less than the current recommended concentration in the U.K of 15 $\mu g ml^{-1}$. As the first portion of the plot in Figure 5.11 was linear, it was

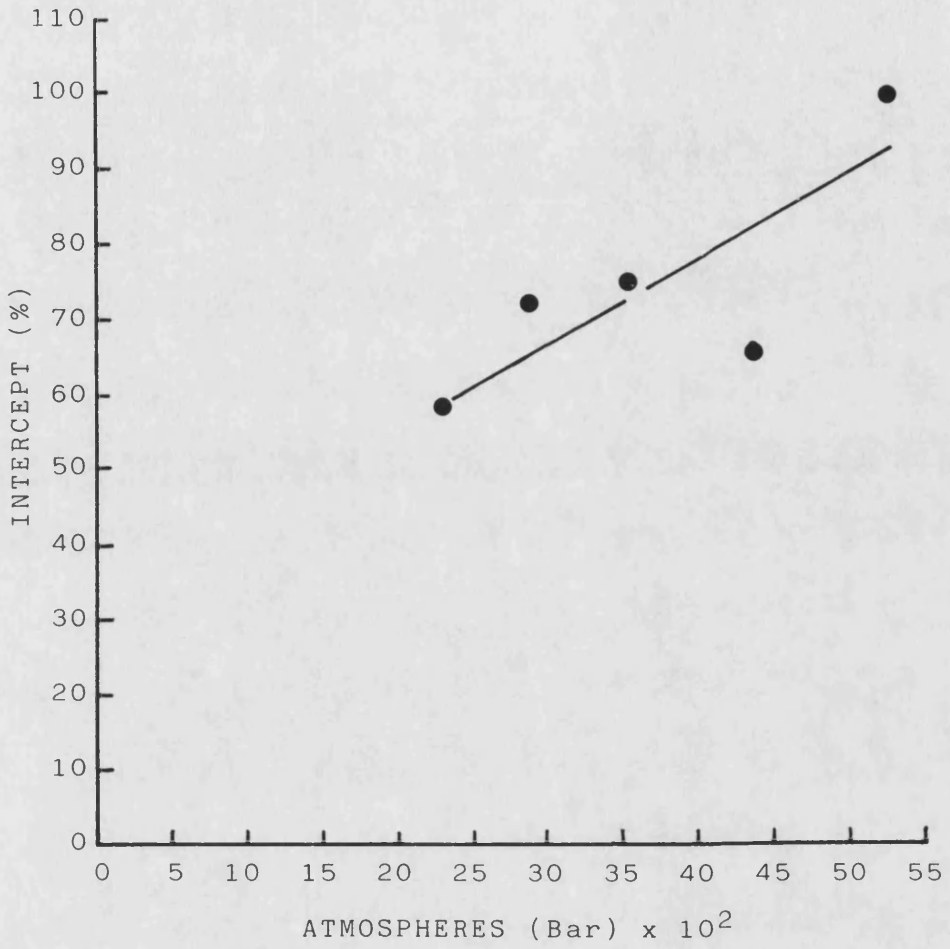


Figure 5.13 Plot of Intercept on Y-axis Against Pressure Difference Between Miniclave 80 Chamber and Atmospheric Pressure for Survivor Curves of Spores of B. stearothermophilus NCIB 8224 Produced on C-limited Medium When Exposed to LTSF at 12 ug/ml Formaldehyde Concentration Over a Range of Temperatures.

decided to determine the concentration exponent (n) using those three points, to see how this value compared to other published values. The plot used to determine this value is illustrated in Figure 5.12. This exhibits a linear relationship as shown by linear regression (Table 5.3), with a correlation coefficient of 99.60%. From this regression, the concentration exponent (n) was calculated to be 1.4. This figure for n is of the same order of magnitude to those quoted for aqueous formaldehyde systems, which are generally stated to have a concentration exponent of 1 (Russell, 1987). The difference is quite likely to be due to only three points being used to calculate the value, which leaves a lot of room for error, and this value would have to be confirmed by further investigation. A figure of 1.4 appears to demonstrate that the concentration of formaldehyde has a similar effect on the rate of inactivation in gaseous systems as in the aqueous ones. Concentration exponent values have been related to the mechanism of lethality (Hugo and Denyer, 1987) and such a similar value obtained for LTSF as for aqueous inactivation appears to suggest the same or a similar lethal mechanism is resposible for spore inactivation in both cases.

It would appear from the results presented here that, over the ranges used in this investigation, the concentration of formaldehyde and not temperature is the critical factor in LTSF. This has good implications for the use of LTSF for very heat labile items, offering a possibility of a reduction in operating temperature from 73°C to 63°C. The concentration of formaldehyde must be determined on a compromise between bactericidal effect required, practicality (such as polymer formation, airing-off times) and safety. With the advent of COSHH (Control of Substances Hazardous to Health) the safety aspect will become more critical, especially in the light of recent

reports on the occupational health hazards of formaldehyde (Wartew, 1983; Pabst, 1987). Nevertheless, this must also be looked at in the light of the known risks of ethylene oxide, for which it is considered as a replacement.

From the studies reported in this chapter, the use of *B. stearothermophilus* NCIB 8224 sporulated on C-Ltd medium as a Biological Indicator for LTSF seems attractive. This organism displays many of the characteristics required for a biological indicator, including simple sporulation medium, ease of sporulation (cleaning), linear inactivation kinetics, relatively high GI and reproducibility between batches. Further work will be required to optimise such factors as recovery conditions (see Chapter 6), recovery media, and presentation to LTSF (carrier system, etc.), before it could become a viable commercial product.

CHAPTER 6

STUDIES ON THE EFFECT OF HEAT TREATMENT OF SPORES EXPOSED TO LTSF, PRIOR TO PLATING ON RECOVERY MEDIA

6.1 INTRODUCTION

Many methods have been proposed to enhance recovery of damaged spores after exposure to sporocidal agents. These methods have included altering recovery media composition (Roberts, 1970; Mallidis and Scholefield, 1987), ultrasonic and abrasive treatments (Gorman *et al*, 1983), pH (Cook and Brown, 1965b), reduced incubation temperatures (Cook and Gilbert, 1968; Harris, 1963) and combinations of these methods (Gorman *et al*, 1983).

During studies on the antimicrobial action of formaldehyde Spicher and Peters (1976;1981) reported on the phenomenon of heat reactivation. Spores which, by using conventional recovery techniques, did not appear capable of forming colonies could be induced to do so by a heat treatment, after formaldehyde inactivation, and prior to plating. In these studies spores of *Bacillus subtilis* were inactivated in a 15% aqueous formaldehyde solution at 20°C for 2 h, resulting in a four log cycle reduction in survivors. The spores were then suspended in a phosphate buffer (pH 7.0) containing 0.1% Tween. This suspension was then maintained at a constant temperature between 50 and 90°C for up to 120 minutes. This treatment resulted in up to a three log cycle increase in survivors. These results reported by Spicher and Peters are of particular interest. If the recovery of spores of the Biological Indicator organism after exposure to LTSF could be enhanced by a heat treatment prior to plating, then it could have serious implications for the use of LTSF as a sterilizing system and also for the Biological Monitoring of LTSF. One of these is that if the Biological Monitor was exposed to heat in the absence of formaldehyde, for example during the elution phase of the Miniclave 80 cycle, it may be that a

higher viable count would be obtained from the Biological Monitor and hence the effectiveness of the LTSF cycle underestimated.

This chapter reports on investigations into whether this heat reactivation effect occurs with spores of *B. stearothermophilus* NCIB 8224 produced on C-limited medium. Firstly, it was necessary to ascertain whether it occurred in aqueous suspension, e.g. under the screening conditions used in Chapter 3, and secondly, more importantly, whether it occurred with spores exposed to LTSF conditions.

6.2 EXPERIMENTAL

6.2.1 Effect of heat treatment at 85°C, 90°C and 97°C on the recovery of spores of *B. stearothermophilus* NCIB 8224, exposed to 0.5% aqueous formaldehyde at 70°C for 60 minutes.

To determine if heat treatment at a range of temperatures had any effect on recovery of spores exposed to aqueous formaldehyde, a series of experiments was conducted. A suspension of spores which had been exposed to formaldehyde for 60 minutes was chosen for heat treatment as almost a 1 log cycle reduction in % survivors had occurred which would allow significant increases to be seen if they occured.

Spores of *B. stearothermophilus* NCIB 8224 produced on C-limited medium were inactivated at 70°C in 0.5% aqueous formaldehyde by the method described in section 3.3.4. After 60 minutes exposure to formaldehyde, a 1 ml sample was taken, and added to 9 ml of 10% glycine solution. This was left to stand at room temperature for 10 minutes. Two tubes containing 18 ml of sterile water were prepared. One was placed in a waterbath (Grant Instruments Ltd) at the heat activation temperature, and left to equilibrate for a minimum of 15

minutes, the other was held at room temperature as the control. 2 ml volumes of the suspension of spores in 10% glycine were added to each tube and thoroughly mixed. At predetermined time intervals, 1 ml samples were taken from each tube, and added to 9 ml of sterile water at room temperature. This was continued up to a maximum sampling time of 120 minutes. All of the samples were then serially diluted, and plated for enumeration of viable spores using the filter method as described in section 3.3.4. This experiment was carried out for three heat treatment temperatures of 85°C, 90°C and 97°C. A 90°C treatment temperature was chosen as this was found to be most effective for *B. stearothermophilus* in the study carried out by Spicher and Peters. The other temperatures were chosen to bracket 90°C with 97°C being the maximum attainable in a waterbath. The results are plotted in Figure 6.1. The results illustrated in Figure 6.1 demonstrate that heat treatment of spores that have been exposed to 0.5% aqueous formaldehyde at 70°C, prior to plating, does lead to an increase in % survivors. Of the three heat treatment temperatures used the 97°C treatment was the least effective, with a maximum increase in % survivors attained after 40 minutes heat treatment followed by a decline. The 85°C treatment was the next most effective with a maximum increase in % survivors after approximately 60 minutes heat treatment, and no further change after that. The 90°C treatment appeared to be the most effective of the three, with a maximum increase in % survivors of $\frac{1}{2}$ a log cycle after 60 minutes of heat treatment These results are discussed in more detail in section 6.3.

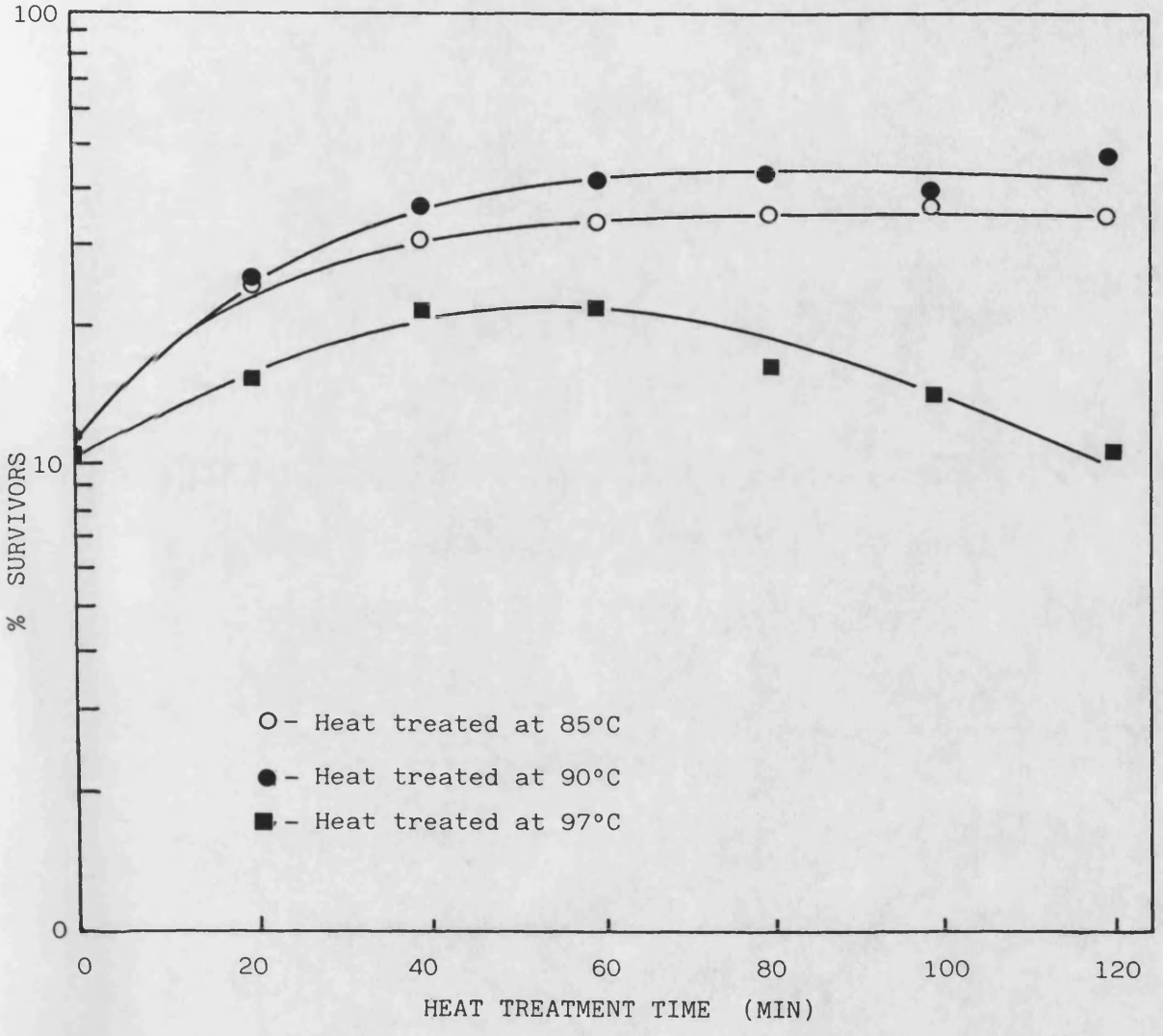


Figure 6.1 Effect of Heat Treatment on Spores of B. stearothermophilus Produced on Carbon Limited Medium After Exposure to 0.5% Aqueous Formaldehyde at 70°C

6.2.2 Effect of a heat treatment of 90°C for 0-120 minutes on the survival of spores of *B. stearothermophilus* after inactivation at 70°C In 0.5% Aq. formaldehyde for 0-120 minutes.

On the basis of the results obtained in section 6.2.1, the optimum heat treatment temperature for reactivation of spores exposed to aqueous formaldehyde is 90°C. This temperature was therefore used to construct survivor curves for untreated and heat treated spores exposed to 0.5% aqueous formaldehyde at 70°C.

Spores of *B. stearothermophilus* NCIB 8224 produced on C-limited medium were exposed to 0.5% aqueous formaldehyde at 70°C by the method described in section 3.3.4 for 0, 60, 90 and 120 minutes. After this time a 1 ml sample was taken and added to 9 ml of 10% glycine solution. This was left to stand for 10 minutes. After this time 2 ml of the spore suspension in 10% glycine was added to 18 ml of sterile distilled water at 90°C. At predetermined time intervals up to a maximum of 120 minutes, 1 ml samples were taken from this volume and added to 9 ml of sterile water at room temperature. Serial dilution was then carried out on all samples and a viable count carried out using the filter method as described in section 3.3.4. The percentage survivors were calculated, and the mean results for two replicates are illustrated in Figure 6.2.

The results illustrated in Figure 6.2 show that for all formaldehyde exposure times, the maximum percentage survivors was attained after 60 minutes heat treatment at 90°C. The magnitude of the maximum increase in % survivors varied greatly, with the 0 minute sample not increasing at all, the 60 minute sample increasing 0.6 of a log. cycle, the 90 minute sample increasing 3/4 of a log. cycle and finally the 120 minute sample increasing 1.5 log cycles. Using the percentage survivors obtained after 0 minutes heat treatment for each

of the four survivor curves illustrated in Figure 6.2, it is possible to construct a survivor curve for spores exposed to formaldehyde and not heat treated. If the same is now done for the number of survivors after 60 minutes heat treatment, a survivor curve for the maximum increase in % survivors for all formaldehyde exposure times, after heat treatment, can be constructed. These plots are illustrated in Figure 6.3. For each of the formaldehyde exposure times, a separate heat treatment experiment had to be carried out, due to the number of sampling points involved. For this reason the survivor curves illustrated in Figure 6.3 are joined with dotted lines.

From the investigations carried out in this section, it is possible to conclude that after exposure of spores of *B. stearothermophilus* NCIB 8224 produced on C-limited medium to aqueous formaldehyde, the percentage of surviving viable spores can be increased by a heat treatment of 60 minutes at 90°C. Furthermore, no further increase in survivors can be achieved by extension of this treatment time up to 120 minutes. Heat treatment of spores which have not been exposed to formaldehyde has no effect on the viable count obtained, demonstrating that the increase in percentage survivors is not due to germination of superdormant spores. The survivor curves illustrated in Figure 6.3 demonstrate that this increase in survivors occurs after heat treatment of spores that have been exposed to aqueous formaldehyde for up to 120 minutes, and that the magnitude of the increase gets larger with spores exposed to formaldehyde for longer times. Finally, it can be concluded that heat treatment of spores after exposure to formaldehyde does not significantly effect the linearity of the survivor curve obtained, but reduces its slope. These results are discussed in more detail in

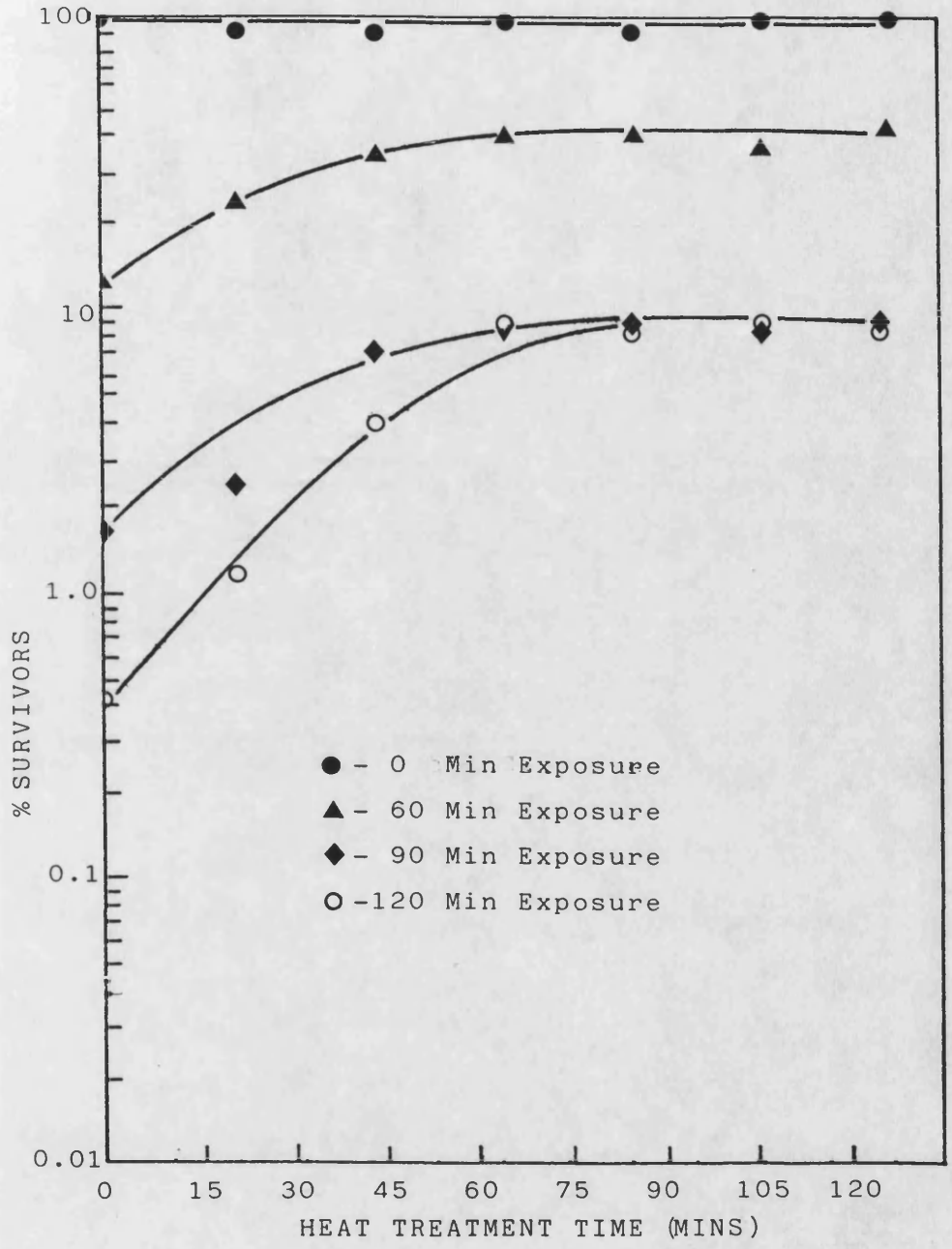


Figure 6.2 Effect of Heat Treatment at 90°C for 0-120 Minutes on the Survival of Spores of *B. stearothermophilus* NCIB 8224 Produced on C-limited Medium after Exposure to 0.5% Aqueous Formaldehyde at 70°C for 0-120 Minutes.

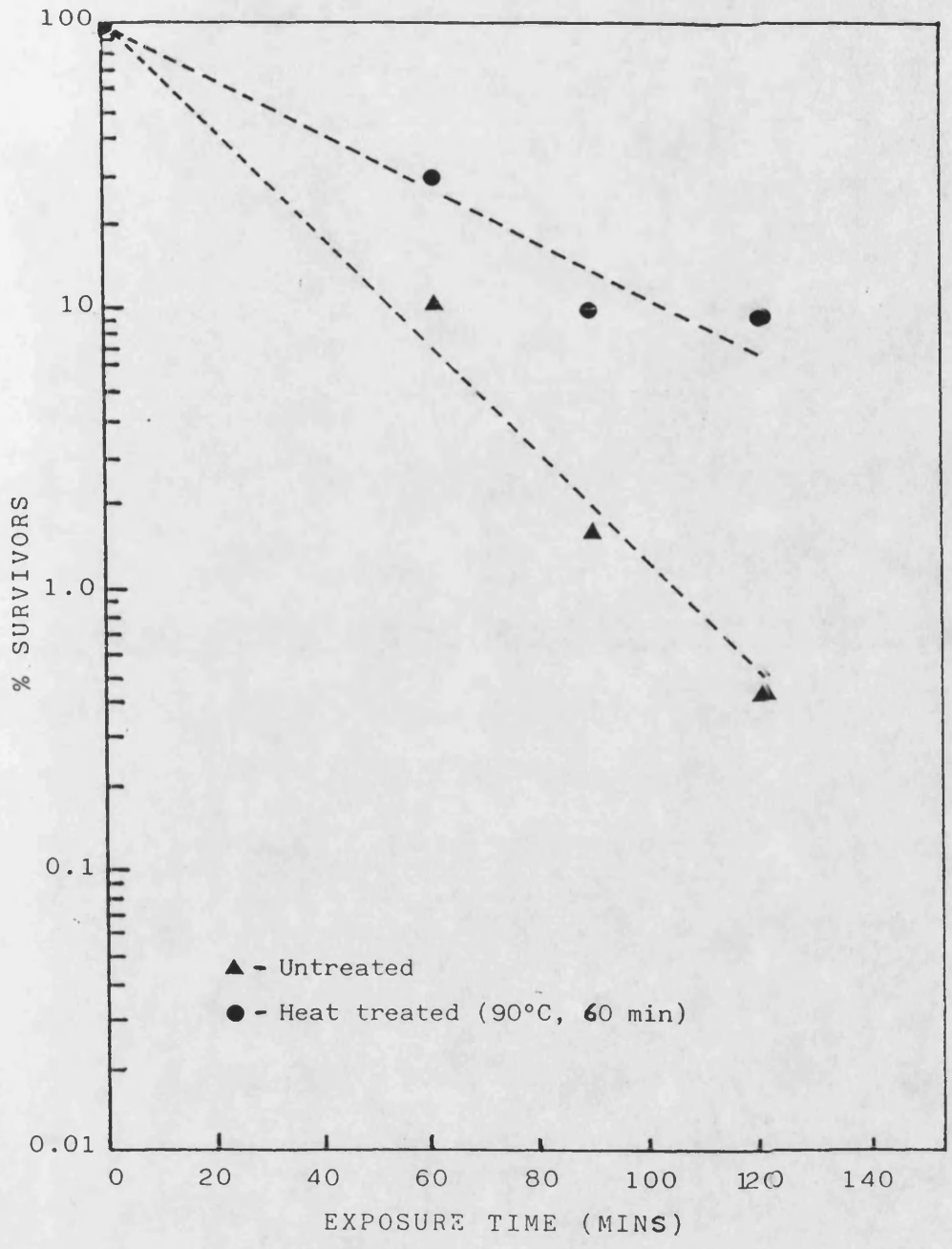


Figure 6.3 Constructed Survivor Curves for Heat Treated and Non-Heat Treated Spores of B. stearothermophilus NCIB 8224 after Exposure to Formaldehyde (0.5% Aqueous) for 0 - 120 minutes at 70°C.

section 6.3.

6.2.3 Effect of extended incubation time at 55°C on survival of spores of *B stearothermophilus* NCIB 8224 after inactivation by 0.5% Aq. formaldehyde at 70 °C.

It has been noted by several researchers that after exposure of spores to inactivating agents, extended incubation of the recovery media can lead to a slight increase in the viable count obtained. This leads to the speculation that the increase in survivors, observed after heat treatment of spores which have been exposed to aqueous formaldehyde, could be an enhanced form of the same effect. To investigate this a comparison of the number of survivors obtained after incubation of the recovery media for 14 days at 55°C against that observed after 60 minutes heat treatment at 90°C, followed by 5 days incubation at 55°C, was carried out.

Spores of *B. stearothermophilus* NCIB 8224 were exposed to 0.5% aqueous formaldehyde solution at 70°C, as described in section 3.3.4. 1 ml samples were taken at predetermined intervals, and added to 9 ml of 10% glycine. This was left to stand for 10 minutes. The samples were then serially diluted and viable counts determined using the filter method described in 3.3.4. The plates were incubated inside plastic boxes for 14 days. A beaker of water was put inside the boxes to humidify the atmosphere to prevent the recovery media drying out. The number of colonies on the recovery media were recorded every day. A plot of survivor curves constructed with the viable counts after 5 days incubation and 14 days incubation is illustrated in Figure 6.4. The survivor curve obtained in section 6.2.2 by heat treatment of spores for 60 minutes at 90°C is also shown in Figure 6.4.

The survivor curves illustrated in Figure 6.4 demonstrate several

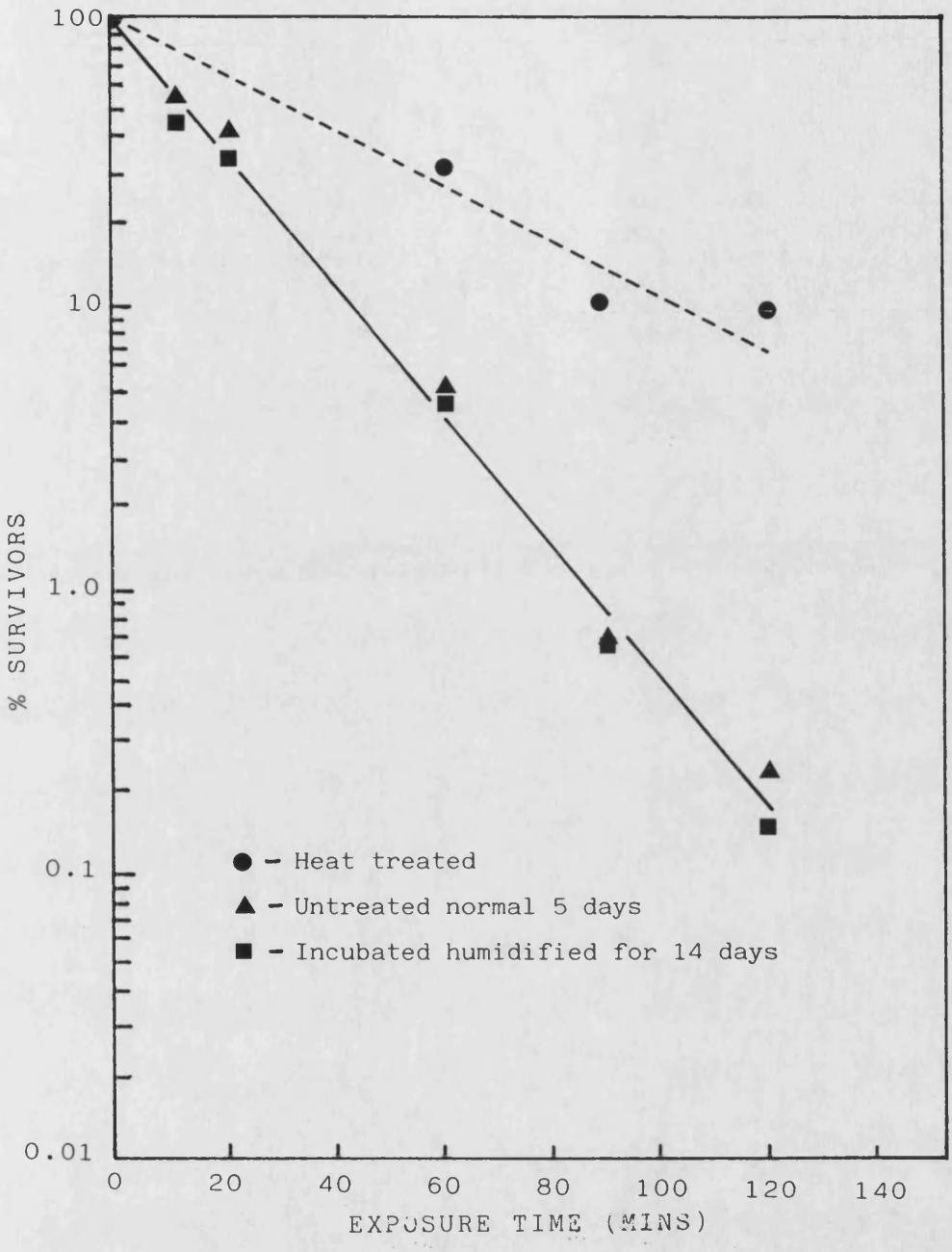


Figure 6.4 Comparison of Heat Treatment and Extended Incubation of Spores of B. stearothermophilus NCIB 8224 after Exposure to 0.5% Aqueous Formaldehyde for 0-120 minutes.

things. Firstly, it is obvious by comparison of the survivor curve constructed from data obtained by extended incubation for 14 days with that of heat treated spores, that it is not possible to obtain as great a number of survivors by extended incubation as that after heat treatment. Secondly, comparing the survivor curve for 5 days incubation with that for 14 days incubation, there appears to be very little difference between them, and is probably within experimental error. This means that incubation of recovery media beyond 5 days is of little benfit, and for this reason, 5 day incubation periods were used in all future work.

6.2.4 Reproducibility of Effect of Heat Treatment at 90°C on spores of *B. stearothermophilus* NCIB 8224 Produced on C-limited Medium after Exposure to LTSF with 12µg/ml Formaldehyde at 73°C.

The studies reported in section 6.2.1 and section 6.2.2 indicate that the heat reactivation effect observed at 20°C by Spicher and Peters (1981) with spores of *B subtilis* exposed to 15% formaldehyde also occurs at 70°C with spores of *B stearothermophilus* NCIB 8224 exposed to 0.5% aqueous formaldehyde. The maximum increase in survivors observed in those experiments was 1.5 log cycles for spores exposed to formaldehyde for 120 minutes. This was a sufficiently significant increase to warrant further investigation.

The next stage of the investigation was carried out using the modified Miniclave 80 (Chapter 4) and spores of *B. stearothermophilus* NCIB 8224 produced on C-limited medium. The aim of these studies was to see if this heat reactivation effect occurred with the organism selected as a potential Biological Indicator for LTSF under the inactivating conditions used in LTSF sterilization, and if so, if the effect was reproducible. For this investigation, LTSF conditions of

73°C and 12 µg/ml formaldehyde were chosen as they were easily attained by the Miniclave 80, and 73°C being the operating temperature for LTSF in the U.K. was relevant.

Test pieces of *B. stearothermophilus* NCIB 8224 produced on C-limited medium were prepared as detailed in section 4.5. These test pieces were then exposed to LTSF conditions at 12 µg/ml concentration of formaldehyde at a temperature of 73°C. The methods described in section 5.2 were used to expose the test pieces to LTSF and to monitor the formaldehyde concentration during the exposure period. After removal from the Miniclave 80, the test pieces were placed into 10 ml of 1% glycine solution and sonicated for 10 minutes. Parallel ten fold dilutions were carried out into sterile distilled water at 90°C for heat treatment and into sterile distilled water at room temperature as a control. These were left to stand for 40 minutes.

In section 6.2.3, a minimum heat-treatment of 60 minutes was demonstrated to give a maximum increase in survivors. However, to carry out investigations using the Miniclave 80, it was necessary to expose the test pieces to LTSF then sonicate and heat-treat them at the same time as taking formaldehyde samples from the Miniclave 80 and assay them by the method described in section 4.3.1.2. Because of the number of samples and timing involved in these procedures, a 40 minute heat-treatment time, though not the optimum, was the most practical.

After the 40 minutes, serial dilutions in sterile distilled water at room temperature were carried out for all samples, and viable counts were ascertained by the spread plate method described in section 5.2. This experiment was repeated three times. Survivor curves were constructed for the heat-treated spores and the controls. A plot, illustrating each of the three survivor curves for heat-

treated spores, and a mean survivor curve for the control with standard error bars calculated as described in Appendix III, is shown in Figure 6.5. Linear regression analysis was carried out on the mean survivor data for the controls and individually on the data for heat-treated spores. The intercepts, slopes and their standard errors and variances for each of the survivor curves are recorded in Table 6.1. The intecepts and slopes from the survivor curves for the heat-treated spores were statistically compared by students t-test analysis. The three intercepts and slopes were compared in pairs in each of the possible combinations (i.e. 1 & 2, 1 & 3 and 2 & 3). The results of these analyses are also shown in Table 6.1.

The survivor curves for the heat treated spores illustrated in Figure 6.5 actually overlay each other, demonstrating their reproducibility, which is confirmed statistically by the t-tests recorded in Table 6.1 showing no difference between any of the intercepts or slopes at a probability level of p = 5%. This means that heat treatment of spores of *B. stearothermophilus* NCIB 8224, after exposure to LTSF, leads to a reproducible increase in survivors compared to spores which are not heat treated. The increase in survivors obtained ranges from 1/2 a log cycle for spores exposed to LTSF for 1 minute to 2 1/2 log cycles for spores exposed for 15 minutes. The linearity of the survivor curves is not greatly affected by heat-treatment except for the 15 minute exposure time, which exhibits a lower surviving percentage than would be expected for a linear survivor curve, which is reflected in the values for correlation coefficients when compared to the controls. Whether this is a true reflection of what is happening could only be demonstrated by repeating the experiments to obtain more data, or repeating the experiments with longer exposure times to LTSF (though this becomes

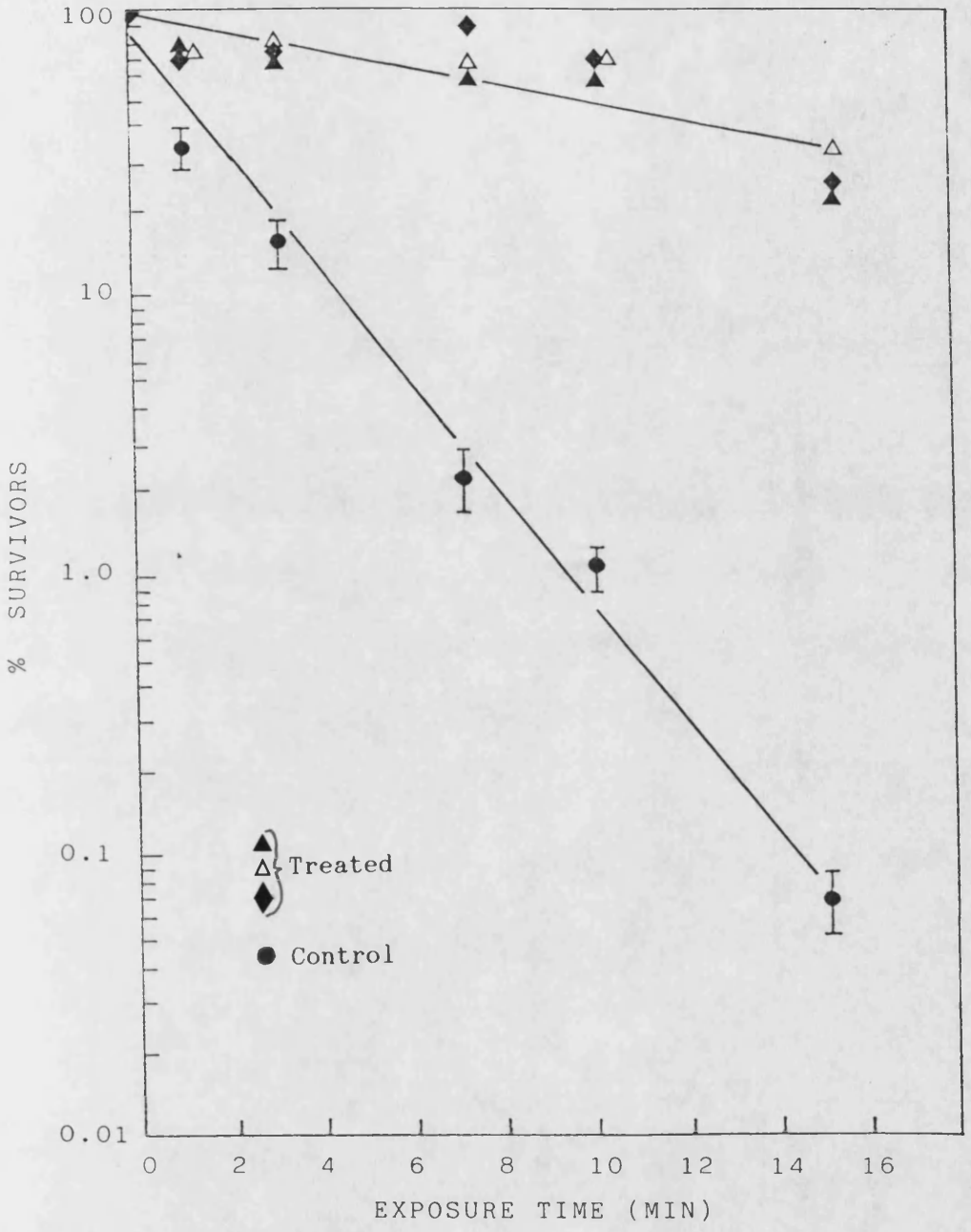


Figure 6.5 Reproducibility of Increase in Survivors Obtained by a Heat Treatment of 90°C for 40 Minutes of Spores of B. stearothermophilus NCIB 8224 Produced on C-limited after Exposure to LTSF with 12 ug/ml Formaldehyde at 73°C

Table 6.1 T-test to compare Slopes and Intercepts Obtained by Linear Regression Analysis of Three Replicate Survivor Curves of Spores of *B.stearothermophilus* NCIB 8224 Heat Treated at 90°C for 40 Minutes After Exposure to LTSF with 12 µg/ml Formaldehyde at 73°C.

Exposure Time (min)	Log Percentage Survivors (1)	(2)	(3)
0	2.00	2.00	2.00
1	1.90	1.84	1.85
3	1.84	1.92	1.79
7	1.86	1.99	1.85
10	1.85	1.83	1.75
15	1.15	1.15	1.48

Regression Data

Replicate	Slope	Standard Error	Variance	Intercept	Standard Error	Variance
1	-0.0265	0.0068	0.00023	1.946	0.054	0.015
2	-0.0434	0.0174	0.00151	2.050	0.139	0.096
3	-0.0441	0.0148	0.00109	2.031	0.118	0.070

t-test

Comparison	t-value (slope)	t-value (intercept)	Degrees of Freedom	t (6) for p = 0.05
1 and 2	0.405	0.31	6	2.45
1 and 3	0.490	0.29	6	2.45
2 and 3	0.014	0.047	6	2.45

difficult for the controls due to there only being 1 x 10^6 spores/test piece). Nevertheless, the study described here demonstrates that not only does heat reactivation of spores exposed to LTSF occur, but also that it occurs reproducibly and to a greater extent than in the aqueous studies described in section 6.2.3, and hence was worth further investigation. Also this study demonstrates that 40 minutes heat treatment at 90°C, though not necessarily the optimum, produces adequate heat reactivation for use in further studies. These results are discussed in greater detail in 6.3

6.2.5 Effect of heat treatment at 90°C for 40 minutes on spores of *B. stearothermophilus* NCIB 8224 produced on C-limited medium after Exposure to LTSF with 12 µg/ml Formaldehyde at Temperatures of 63°C to 83°C.

The study described in the previous section demonstrated that heat reactivation of spores of *B. stearothermophilus* NCIB 8224 which have been exposed to LTSF was possible, and that this reactivation was reproducible using a heat treatment of 40 minutes at 90°C. The next part of this investigation was to demonstrate the effect of heat treatment on survival of spores which had been exposed to LTSF at a range of exposure temperatures.

Test pieces of *B. stearothermophilus* NCIB 8224 produced on C-limited medium were prepared as detailed in section 4.3.2. These test pieces were then exposed to LTSF conditions at 12 µg/ml concentration of formaldehyde at temperatures of 63°C, 68°C, 73°C, 78°C and 83°C. The method for exposing the test pieces, to LTSF conditions and the procedures for monitoring the formaldehyde during exposure were the same as described in section 5.2. When the test pieces were removed from the Miniclave 80, they were sonicated in

10 ml of 1% glycine solution for 10 minutes. Parallel ten-fold dilutions were carried out into sterile distilled water at 90°C for heat treatment and into sterile distilled water at room temperature as a control. These were left to stand for 40 minutes and further serial dilution of each were carried out in sterile distilled water at room temperature. These dilutions were plated by the spread plate method described in section 5.2 and the colonies counted after 5 days incubation at 55°C. At least three replicate experiments were conducted at LTSF inactivation temperatures of 63°C, 68°C, 73°C, 78°C and 83°C. Mean survivor curves were constructed for the control and for the spores heat treated after exposure to formaldehyde for each of the inactivation temperatures. These curves were calculated using linear regression analysis and plotted with standard error bars. The regression analysis and standard error bars were calculated as described in Appendix III, and the data are illustrated in Figures 6.6 to 6.10.

The first conclusion that can be drawn from the data illustrated in Figures 6.6-6.10 is that spores of *B. stearothermophilus* NCIB 8224 can be reactivated by a heat treatment at 90°C after exposure to LTSF conditions. Also a comparison of the survivor curves for spores exposed to formaldehyde only with those for spores which were heat treated after the exposure, demonstrates that the linearity of the survivor curves is not unduly affected by the heat treatment. The major effect of the heat treatment is to reduce the slope of the survivor curve. The good reproducibility of the survivor curves of heat treated spores at all five temperatures (as demonstrated for LTSF at 73°C in section 6.2.4) is indicated by the standard error bars on the data points of the survivor curves. The ratio of the k values of survivor curves of heat treated spores to those of spores

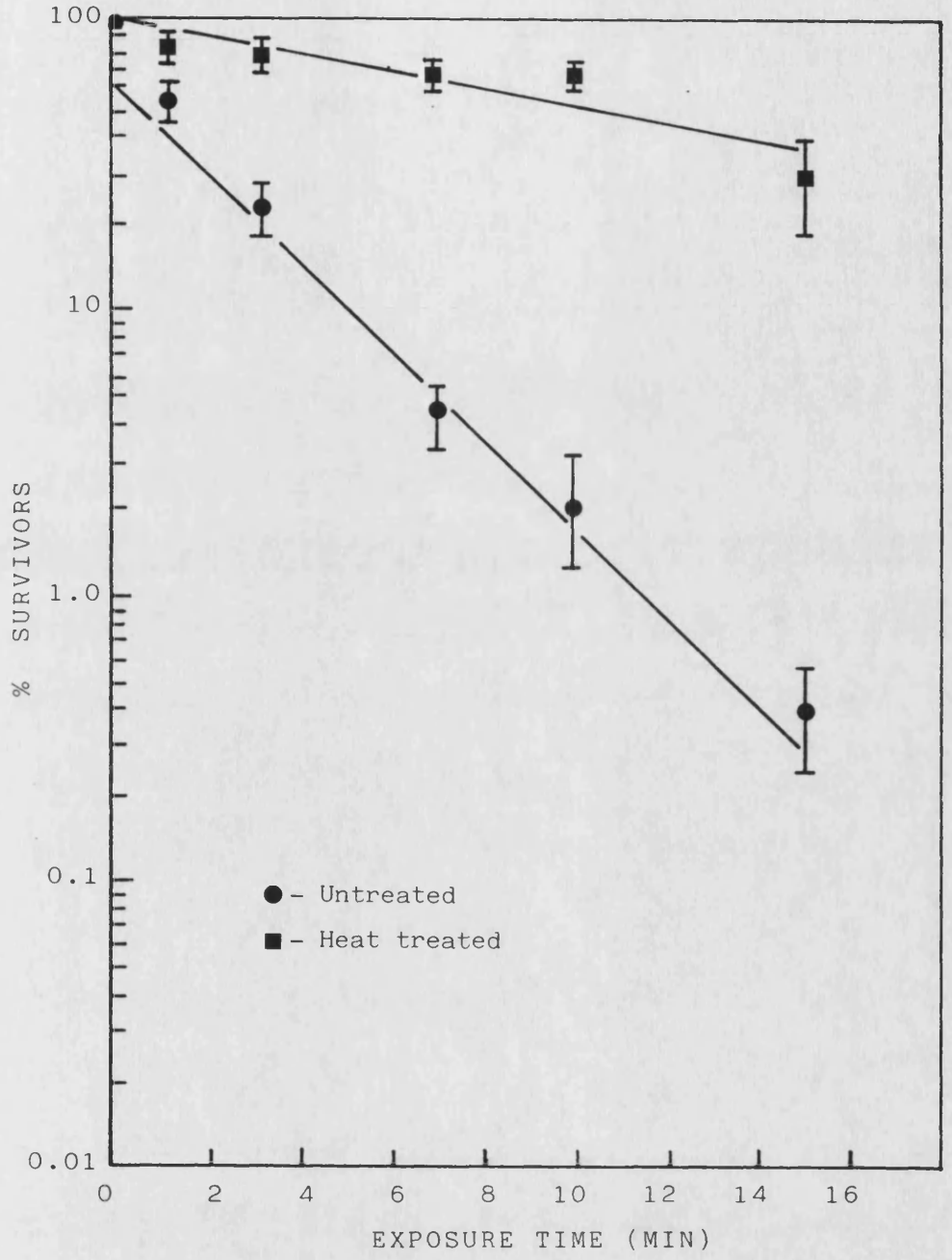


Figure 6.6 Effect of Heat Treatment at 90°C for 40 minutes on Spores of B. stearothermophilus NCIB 8224 after Exposure to LTSF at 12 ug/ml Formaldehyde at 63°C.

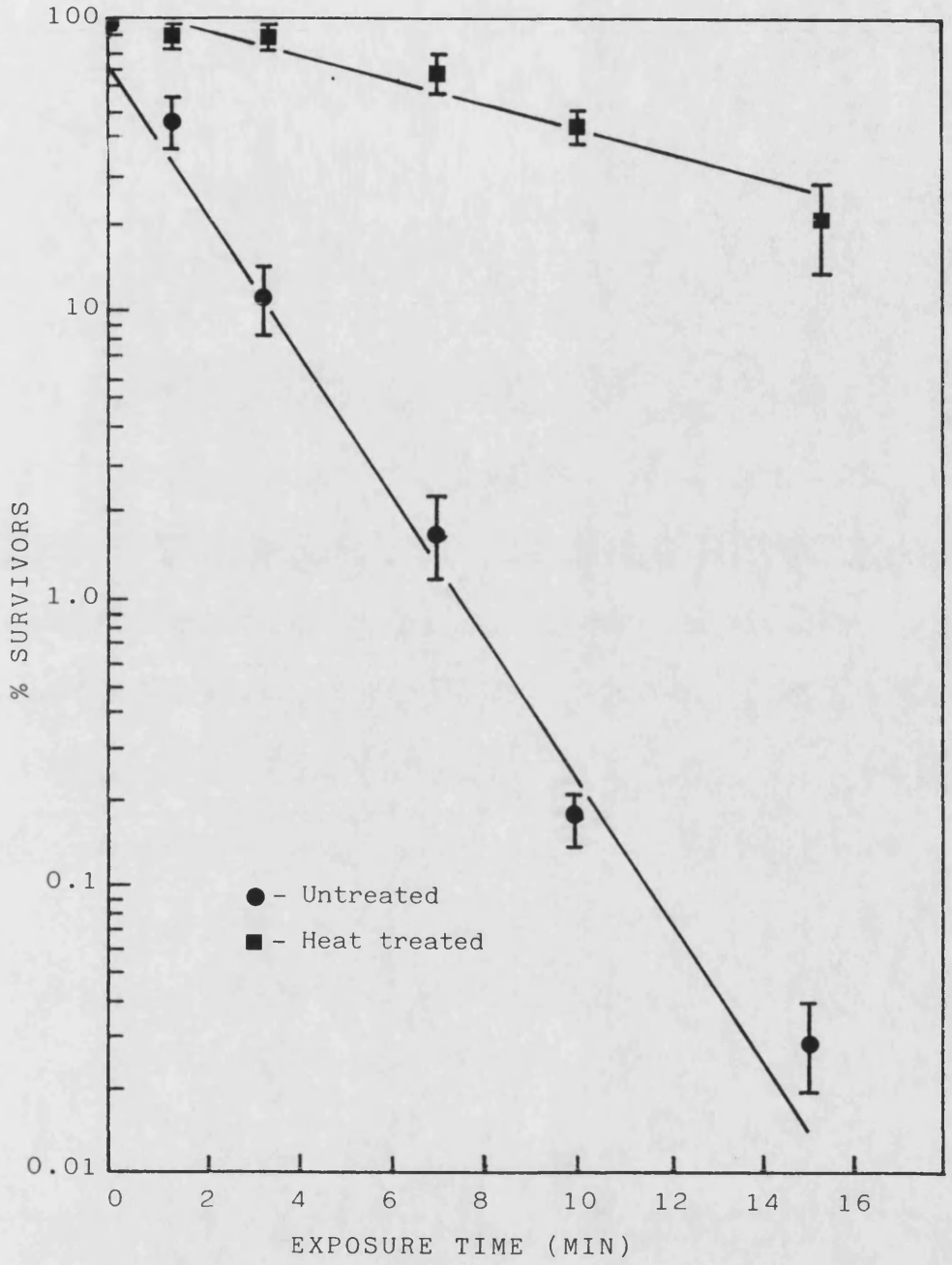


Figure 6.7 Effect of Heat Treatment at 90°C for 40 minutes on Spores of B. stearothermophilus NCIB 8224 after Exposure to LTSF at 12 ug/ml Formaldehyde at 68°C.

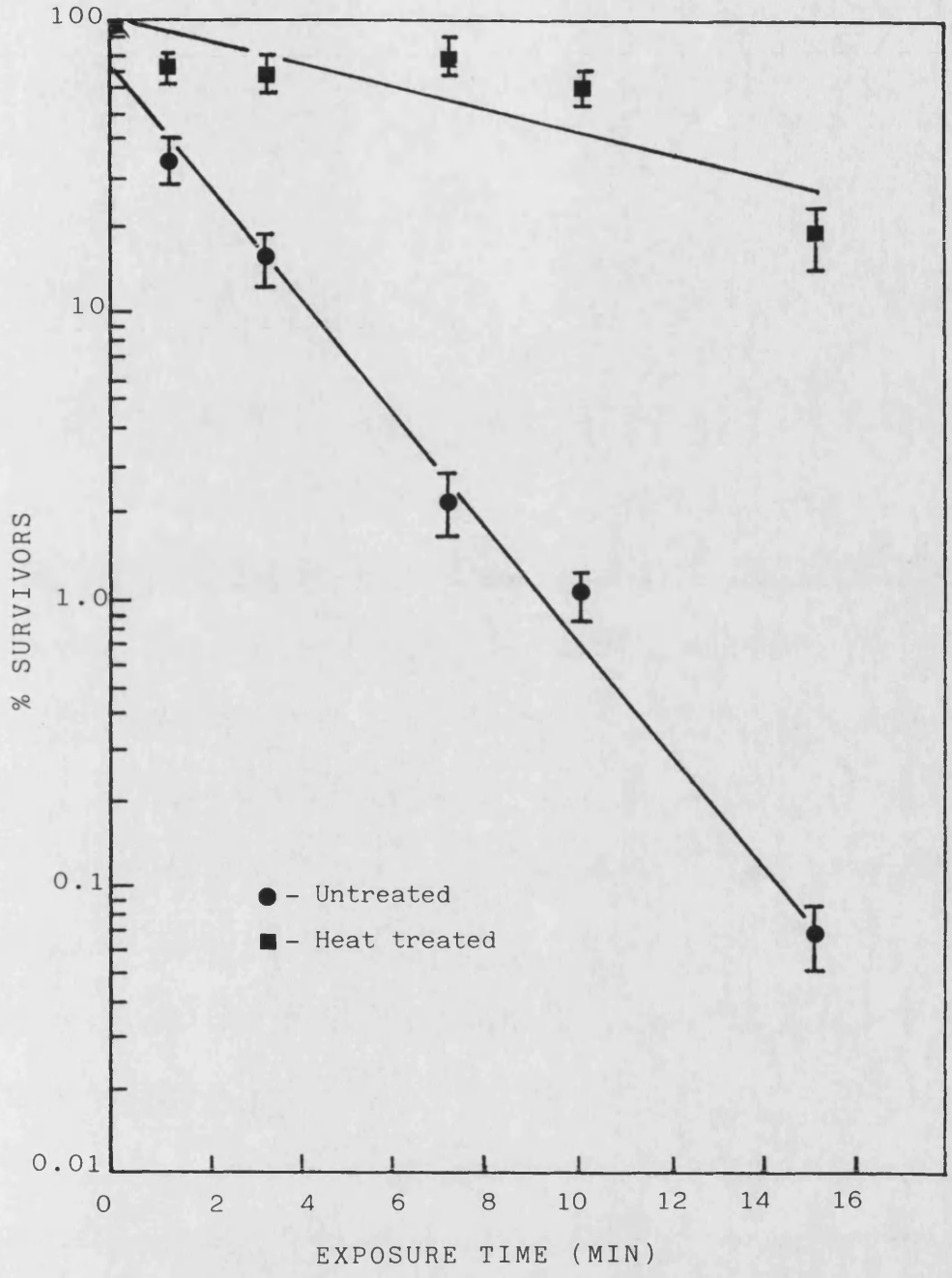


Figure 6.8 Effect of Heat Treatment at 90°C for 40 min on spores of B. stearothermophilus NCIB 8224 Exposed to LTSF with 12 ug/ml formaldehyde at 73°C.

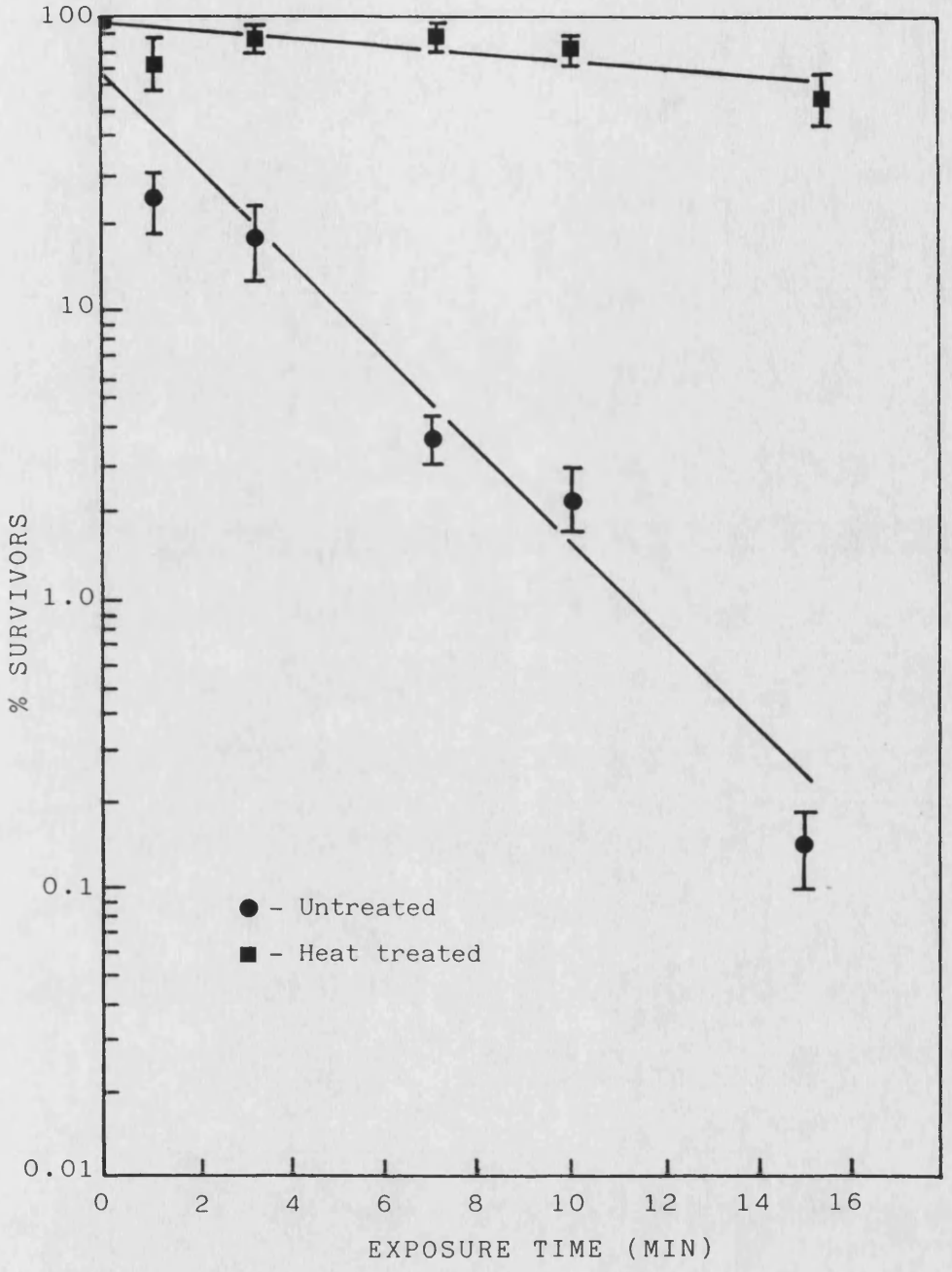


Figure 6.9 Effect Of Heat Treatment at 90°C for 40 minutes on Spores of B. stearothermophilus NCIB 8224 after Exposure to LTSF at 12 ug/ml Formaldehyde at 78°C.

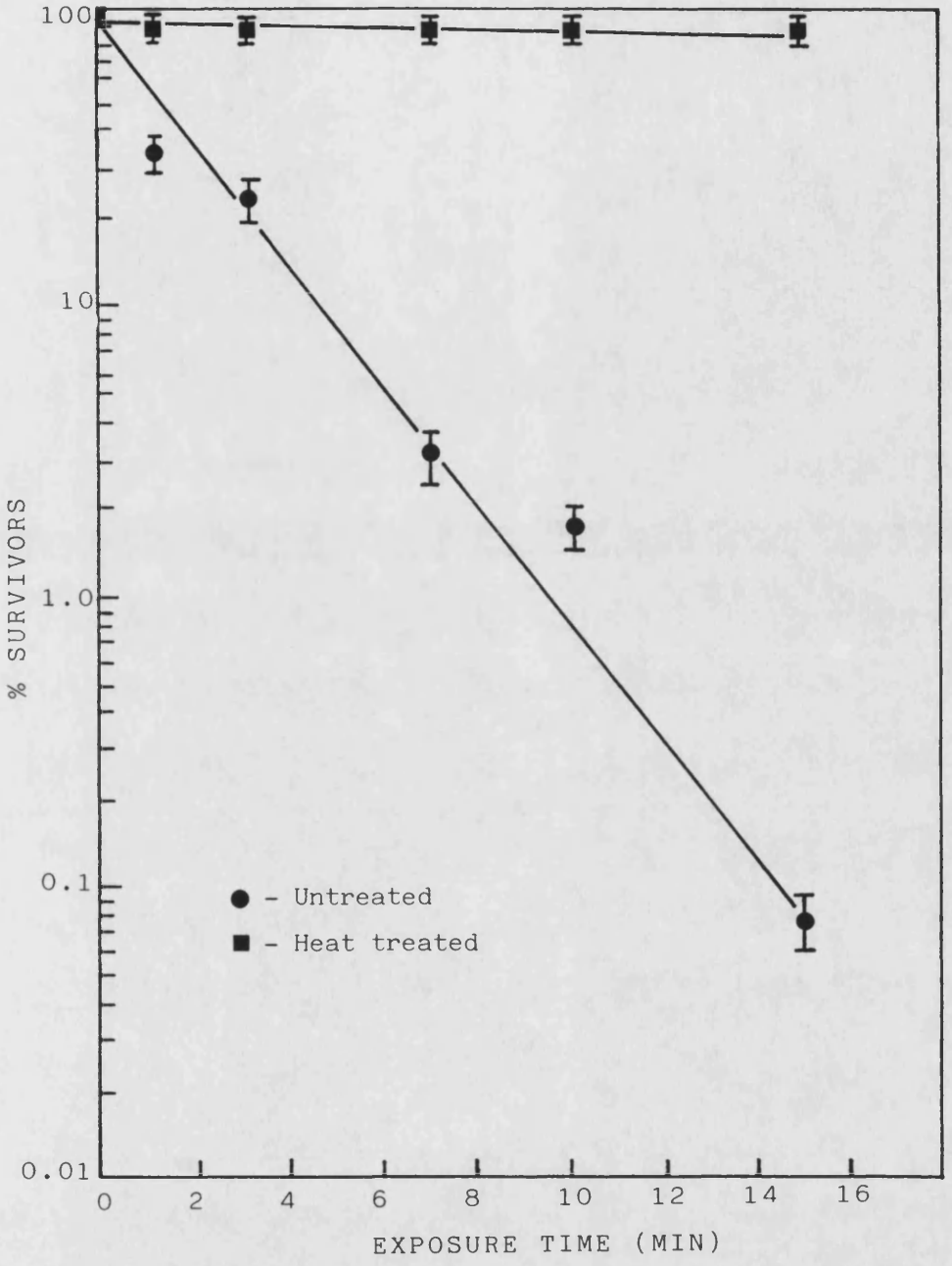


Figure 6.10 Effect of Heat Treatment at 90°C for Minutes on Spores of B. stearothermophilus NCIB 8224 after Exposure to LTSF at 12 ug/ml Formaldehyde at 83°C.

not heat treated are shown in Table 6.2. These ratios can be used to compare the effect of heat treatment at each of the LTSF exposure temperatures. Firstly this comparison shows that in all cases the

Table 6.2 Ratio of Inactivation Rate Constants (k values) of Heat Treated Spores to Those of Spores not Heat Treated After Exposure to LTSF at 12 $\mu g ml^{-1}$ Formaldehyde Concentration at 63°C to 83°C

Inactivation Temperature (°C)	k values (min^{-1}) Untreated	Heat Treated	Ratio (Untreated/Treated)
63	0.368	0.057	6.46
68	0.570	0.098	5.82
73	0.477	0.087	5.48
78	0.378	0.028	13.50
83	0.483	0.0069	70.00

ratio of the k values is greater than 1 indicating that heat treatment resulted in a reduction in k value (i.e. an increase in survivors) for all of the LTSF exposure conditions. This comparison also indicates that the amount of reactivation obtained by heat treatment of spores exposed to LTSF at higher temperatures (e.g. 78°C and 83°C) is greater than that obtained with spores exposed to LTSF at lower temperatures. These results and their implications are discussed in more detail in section 6.3.

6.2.6 Effect of delay in heat treatment on the magnitude of increase in number of survivors after inactivation by LTSF at 12 µg/ml formaldehyde at 73°C

It has been suggested that the sporocidal effect of formaldehyde has two stages (Hurrell, 1988). Firstly, the formaldehyde reversibly reacts with components of the spore with weak bonds. Secondly these weak bonds strengthen, and the formaldehyde becomes irreversibly attached. Assuming this theory to be correct, a possible explanation for the heat reactivation effect observed in the studies carried out in section 6.2.4 is that the heat treatment is breaking the reversible formaldehyde bonds before stronger bonds can form. Breaking of the bonds in this way would then allow the formaldehyde to leave the spore by simple diffusion, resulting in a lower "kill" and survivor curve slope. To test this theory, it was decided to expose spores to LTSF, then delay the heat treatment for up to 21 hours after the exposure to LTSF and observe the effect on the survivor curves obtained.

Test pieces of *B. stearothermophilus* NCIB 8224 produced on C-limited medium were prepared as detailed in section 4.3.2. These test pieces were exposed to LTSF conditions at 12 µg/ml formaldehyde concentration and 73°C. The method of exposing the test pieces to LTSF and of monitoring the formaldehyde concentration during the exposure were the same as described in section 5.2. When the test pieces were removed from the LTSF conditions, they were sonicated in 10 ml of diluent for 10 minutes. The diluent was 1% glycine solution in the first study, and sterile distilled water in the second. These were then stored at 25°C for 1, 6 and 21 hours. After these times 2ml samples were taken and added to 18ml of sterile distilled water

at 90°C for heat treatment, and into sterile distilled water at room temperature as a control. These were then left for 40 minutes after which serial dilution of both was recommenced into sterile distilled water at room temperature. The number of survivors was determined by the spread plate technique described in section 5.2. Survivor curves were constructed for the heat treated spore suspensions and the controls for each of the storage times. These are illustrated in figures 6.11 and 6.12. As the results for 1, 6 and 21 hours storage were very close, the 6 hour points were omitted in these survivor plots for clarity.

Several conclusions can be drawn from the results illustrated in Figures 6.11 and 6.12. Firstly the results clearly illustrate that storage of spores which have been exposed to LTSF, before heat treatment is carried out, has no effect on the increase in survivors obtained by the heat treatment. Secondly, comparison of the survivor lines for both the controls and the heat treated spores in Figures 6.11 and 6.12 reveals no discernible differences. This suggests that the use of 1% glycine as an inactivator for any formaldehyde carried over on the test piece is not necessary. This may be due to only trace amounts of formaldehyde adhering to the test pieces during the LTSF process, which could be easily be diluted to non-sporicidal levels by sterile distilled water alone. These results are discussed in more detail in section 6.3.

6.3 DISCUSSION

The work carried out by Spicher and Peters (1976; 1981) dealt with an aqueous system. This system was based on a high concentration of formaldehyde (15%) and a low temperature of inactivation (20°C), and *B. subtilis* spores rather than *B. stearothermophilus*. Therefore the

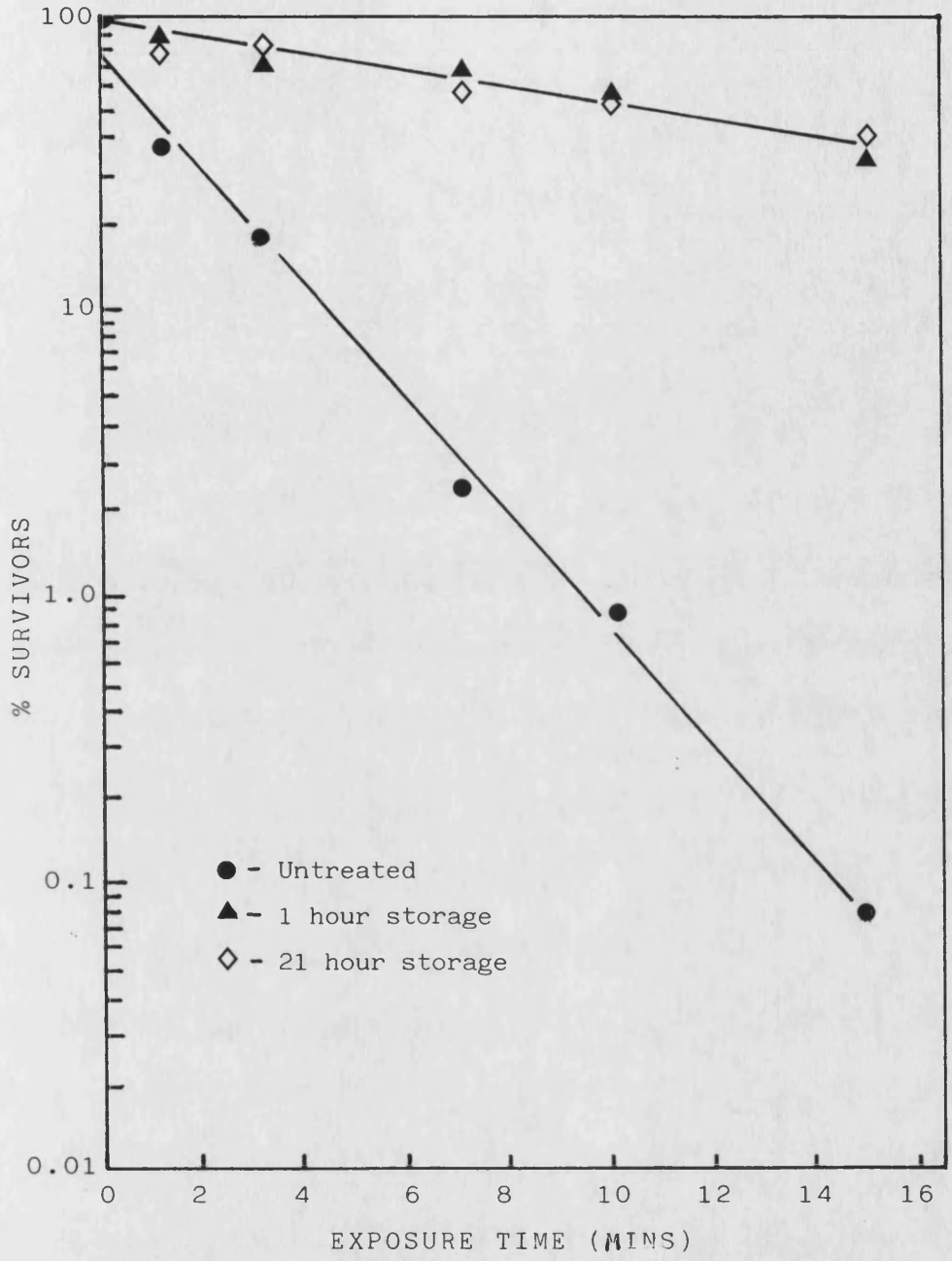


Figure 6.12 Effect of Storage of Spores of B. stearothermophilus NCIB 8224 in Sterile Distilled Water on the Heat Reactivation Obtained by Heat Treatment at 90°C for 40 Minutes.

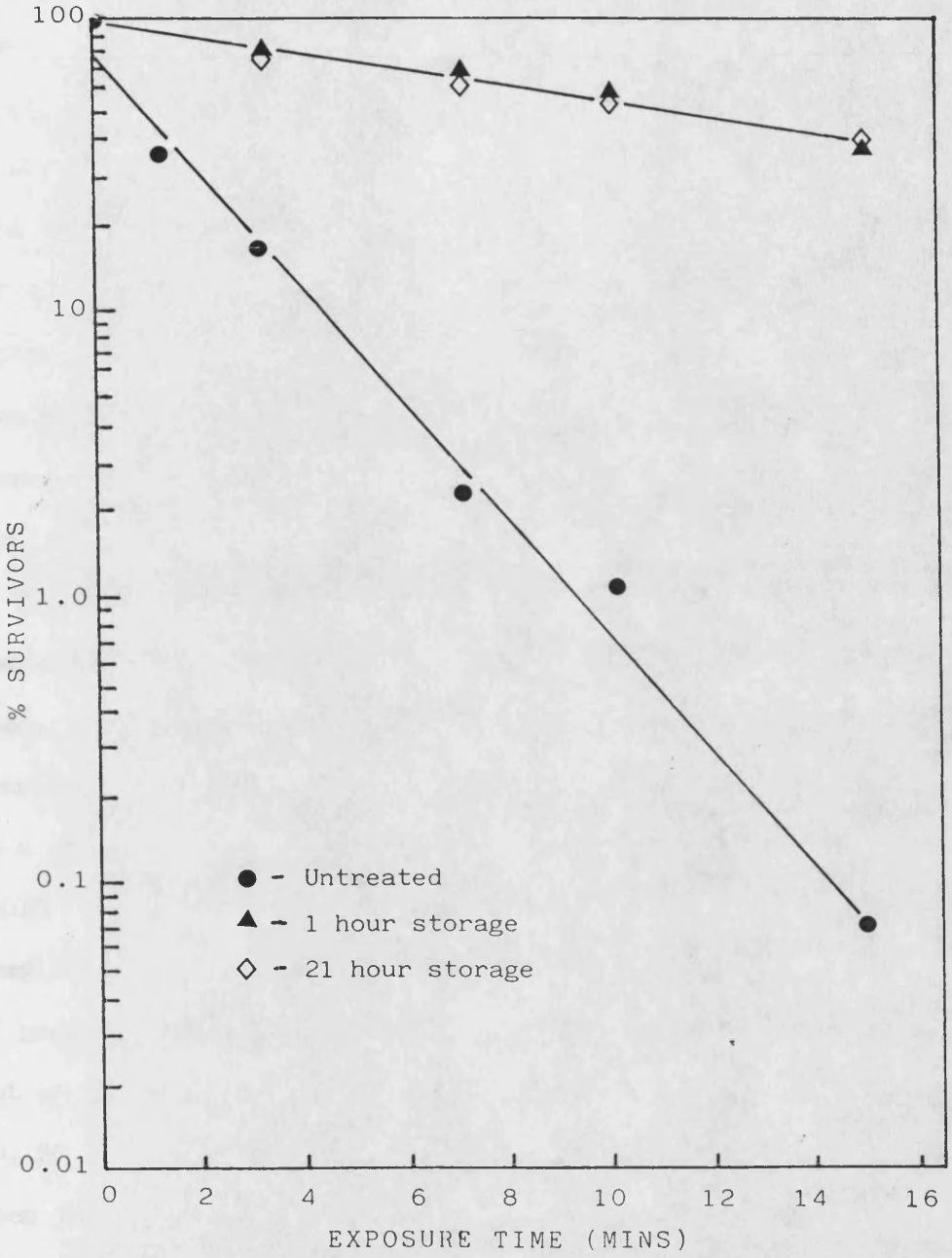


Figure 6.11 Effect of Storage of Spores of B. stearothermophilus NCIB 8224 in 1% Glycine Solution on the Heat Reactivation Obtained by Heat Treatment at 90°C For 40 Minutes.

first part of this investigation was designed to discover if the effect of heat reactivation described by them could occur under the screening conditions used in section 3.3.4, that is 0.5% aqueous formaldehyde at 70°C. The results of this study were conclusive that this effect did occur. The results in Figure 6.1 demonstrate that for a sample of spores inactivated in 0.5% formaldehyde at 70°C the optimum treatment time and temperature is 60 minutes at 90°C. This gave a maximum increase in apparent survivors from 11% to approximately 35%, about 1/3 of a log increase. This result was conclusive enough to warrant further investigation.

The next part of the investigation was to treat a whole range of samples exposed to 0.5% formaldehyde at 70°C for different lengths of time. From this, it was hoped to construct two survival lines, an untreated and heat treated one, to see the effect of heat treatment on a whole survival experiment. The most desirable way to do this would be to carry out a survival experiment, then treat all the time samples to construct the curve. Unfortunately, because of the amount of practical problems this would involve, it was necessary to carry out an individual experiment for each point. This was done for 0, 60, 90 and 120 minute exposure samples, the results of which can be seen in Figure 6.2. Taking the percentage survivors for 0 minutes heat treatment it was possible to construct a "Pseudo survival curve" for spores not heat treated, and using the 60 minute points plot a "pseudo survival curve" for heat treated spores. These plots can be seen in Figure 6.3. This graph illustrates that the heat reactivation effect occurs across the whole range of exposure times. However, the longer the spores were exposed to formaldehyde, the greater the increase caused by heat treatment, though in none of these studies was the reduction in survivors ever completely

reversed. This means that irreversible (by this technique) inactivation is still occurring, and heat treatment has just reduced the slope of the survivor curve. An important point to note is that spores not exposed to formaldehyde (0 minutes on Figure 6.2) exhibited no increase at all when heat treated. This demonstrates that this effect is not a result of heat activation of the super-dormant spores in the suspension, of which there are approximately 30-40%.

It has been reported by several workers that after inactivation experiments, longer incubation times allowed the recovery of more survivors, though often only a few. A study was undertaken to discover whether an extended incubation time (up to 14 days) could recover the same amount of survivors as that obtained in the heat treatment experiment. The results of this study (Figure 6.4) illustrate that this length of time would not reproduce the survivor percentages obtained by heat treatment, and in fact, very little increase in survivors (<0.1 log cycle) was obtained beyond the fifth day of incubation. This is in agreement with the results of Ortenzio (1966), where extended incubation up to 21 days could not match the increase in surviving percentage of spores caused by a heat treatment of 80°C for 20 minutes.

Until this point all the work, including that of Spicher and Peters (1981), has involved the use of aqueous formaldehyde for inactivation of spores. Though from a scientific point of view it is interesting, it does not necessarily have implications for LTSF conditions. The second part of this study was therefore designed to find out whether or not the heat reactivation effect would occur under controlled LTSF conditions and if it did was it reproducible. This investigation involved the exposure of test pieces of

B. stearothermophilus NCIB 8224 to 12 µg/ml at 73°C. After heat treatment at 90°C for 40 minutes, survivor curves for the control, and the heat treated spores were constructed. The treatment temperature was chosen as being close to the optimum found in the aqueous experiments. The time of 40 minutes was a compromise between a convenient treatment time for the experiment, and the optimum of 60 minutes from the aqueous experiments. In fact 40 minutes caused maximum recovery in all but the 120 minute inactivation sample in the aqueous study, and even this sample was very little below maximum (Figure 6.2). The results illustrated in Figure 6.5 demonstrate that heat reactivation does occur with spores exposed to LTSF conditions. Not only does it occur, but by using t-tests to compare the intercepts and slopes of the curves (obtained by linear regression), for the heat treated spores it was demonstrated that there is no significant difference between these lines, and therefore the effect is reproducible. The amount of heat reactivation exhibited in this study was greater that that obtained in the aqueous studies. It has been suggested (Hurrell, 1988) that heat reactivation could be a consequence of breaking weak bonds, formed by formaldehyde, with the spore prior to permanent bond formation. If this was so, it would follow from these results that the bonds formed during inactivation of spores with aqueous formaldehyde are stronger than those formed in LTSF, or alternatively form permanent bonds quicker than is the case for LTSF inactivation of spores.

The next part of this investigation was designed to demonstrate the effect of heat treatment of spores which had been exposed to LTSF at different exposure temperatures. Spores were exposed to LTSF at a formaldehyde concentration of 12 µg/ml at temperatures between 63°C and 83°C in the modified Miniclave 80 described in Chapter 4. These

were then sampled, and half of the samples serially diluted at room temperature then incubated at 55°C for 5 days as controls. The other half were heat treated at 90°C for 40 minutes, before being serially diluted and incubated on recovery media for 5 days at 55°C. The results of these experiments are illustrated in Figures 6.6-6.10. The inactivation rate constants (k values) of the survival curves for heat treated and untreated spores were calculated, and these plus the ratio of k values for untreated to heat treated spores are shown in Table 6.2. The ratios of k values shown in Table 6.2, all greater than 1.0, demonstrate that an increase in survivors was obtained for spores that were heat treated after exposure to LTSF at all temperatures between 63°C and 83°C. The magnitude of the increase in survivors (decrease in k value) varies with the LTSF exposure temperature. Spores that were exposed to LTSF at higher temperatures exhibited the greatest increase in survivors. Spores exposed to LTSF at 63°C to 73°C but not heat treated exhibited approximately six-fold greater k values than those for spores that were heat treated. Spores which were exposed to LTSF at 78°C and 83°C exhibited much greater reductions in k values, with the spores that were not heat treated having 13 fold and 70 fold greater k values respectively, than those which were heat treated. This increase is more dramatic than that obtained with the aqueous formaldehyde inactivation experiments. The 63°C LTSF heat treated line still has a projected D-value of approximately 36 minutes as opposed to a D-value of 5 minutes if not heat treated. The 83°C LTSF survivor curve has altered from an untreated D-value of just under 5 minutes, to a heat treated value which appears almost infinite, as the survival line appears to level out at approximately 90% survivors.

Other workers have reported on increases in the number of spores

recovered, after inactivation, by heat treating the spores prior to plating. Spicher and Peters (1981) have already been mentioned, however they were not the first. Ortenzio (1966) reported that recovery of spores after exposure to constant boiling point hydrochloric acid, could be increased by a 20 minute heat treatment at 80°C. It has been reported that spores of *B. subtilis,* retarded in their growth by hydrogen peroxide treatment, could be reactivated by heat treatment at 80°C for 2-10 minutes. Gorman *et al* (1983) reported that spores of *B. subtilis* could be reactivated, after inactivation by 2% alkaline glutaraldehyde, by heat treatment in phosphate buffer at 50°C to 90°C for up to 120 minutes. More recently Power *et al* (1989) demonstrated increases in survivors, after exposure to 2% alkaline glutaraldehyde, by heat treatment, the most effective temperature being 57°C for 30 minutes. In these cases increases in the numbers of survivors after lethal treatment has been reported; however, except in the case of Spicher and Peters, none reported increases of the magnitude reported here. Particularly in the case of heat treatment after inactivation by LTSF at 83°C, only 10% of the spores seem to be irreversibly inactivated. It is possible however, that even this last 10% may be recovered. This could be done by either optimising the heat treatment for LTSF, or a combination of other recovery techniques such as altering recovery media composition (Roberts, 1970), optimising pH (Cook and Brown, 1965b), abrasive methods (Gorman *et al*, 1983), alkali treatment (Power *et al*, 1989) or coat removal techniques (Gorman *et al*, 1983).

Various theories have been put forward to explain this phenomenon. One of the most likely ones is that the heat treatment is stimulating germination of the dormant spores in the population (approximately 30-40% in the case of NCIB 8224 sporulated on C-limited medium).

This is a widely known phenomenon with *B. stearothermophilus* (Shull and Ernst, 1966; Cook and Brown, 1965a). This phenomenon has also been observed in this report 3.3.4.2. The fact that the heat-treated samples give recovery above that of the projected zero count could suggest this. It was suggested in Section 5.3 that these lower intercepts were due to loss of spores. Therefore if this was true, heat treated samples could only reach counts of 90% by activation of some of the dormant spores. However, the results illustrated in Figure 6.2 clearly illustrate that no heat activation occurs at the heat treatment temperature used in this study, therefore it appears that this cannot be the explanation, and that no, or at least not as many, spores were lost off the carriers as suggested in 5.3. Other explanations have included suggestions that heat treatment may activate unknown systems capable of repairing or bypassing damage (Adams, 1978), or disrupting the spore coat, allowing access of germinants to the germination receptor (Power *et al*, 1989). The latter of these is not considered to be a likely explanation as Russell (1982) stated it was unlikely to have much effect on spore coats. So one of the most likely explanations is that the heat treatment is removing some of the formaldehyde from the spore coat by diffusion. It has been suggested that formaldehyde first forms labile bonds in the spore, leading to more permanent ones after a period of time (Hurrell, 1988). If this was so, it is likely that delay in heat treatment after inactivation in formaldehyde would cause less increase in the numbers of survivors recovered.

The final part of this study was carried out to demonstrate the effect of delaying the heat treatment on the number of survivors obtained. Two studies were carried out. In the first, test pieces were exposed to LTSF at 12 µg/ml and 73°C, then they were put into 10

ml of 1% glycine. In the second study test pieces were exposed to LTSF as with the first, but were then transferred to sterile distilled water instead of 1% glycine. Both of these were then put into a 25°C incubator for up to 21 hours. The results shown in Figures 6.11 and 6.12 demonstrate that there is no visible effect on the normal or heat treated survival curves by storage prior to recovery. The survival curves for samples taken at 6 hours were omitted for clarity as they overlay the results for the other two samples. Two observations can be made about these results. The first is that there is no apparent difference between the survivor curves for non-heat treated spores where 1% glycine was used to inactivate an formaldehyde carried over on the test piece and those where sterile distilled water were used instead. This implies that glycine is not required for the amounts of formaldehyde carried over from LTSF conditions. This is unsurprising as the amount of formaldehyde carried over would be minute, and formaldehyde has little sporocidal activity at room temperatures. The second observation is that if the heat treatment effect is due to removal of formaldehyde from the spore coat, then storage in 1% glycine solution or water at 25°C does not stop this from occurring. This proves that if this is the case, no permanent bonds are formed within this time frame, and that spores are recoverable by heat treatment techniques and not permanently inactivated. This would appear to suggest that the sporocidal mechanism may not take place until the spore proceeds to germinate. A possible way of determining this would be to germinate the spores after inactivation by formaldehyde, then heat treat them. It would be possible then to pinpoint the time at which the inactivation of the spores becomes irreversible by this technique. A germination solution, suggested by Russell (1989) was

tried unsucessfully on spores of *B. stearothermophilus* NCIB 8224. This solution was unsuccessful even in spores that had not been exposed to formaldehyde. This is unsurprising as it has been reported that stimulation of germination of *B. stearothermophilus* can only be achieved by heat treatment at above 100°C.

CHAPTER 7

CONCLUDING DISCUSSION AND SUGGESTIONS FOR FURTHER WORK

7.1 CONCLUDING DISCUSSION.

The search for a reliable method of low temperature sterilization which does not leave tissue irritants in the sterilized load has become urgent. This is due to the increasing use of sophisticated temperature-sensitive materials and instruments. One possible method, gamma irradiation, is slow, expensive, and has undesirable effects on some plastics (Woolston, 1990). Another, ethylene oxide, requires a long degassing period to remove toxic residues, and also requires very stringent safety precautions due to its explosiveness and toxicity. Low temperature steam, at temperatures between 60°C and 80°C, combined with variable amounts of formaldehyde vapour is considered to be safer and leave less toxic residues. Reports of LTSF's effectiveness have varied, however (Alder and Gillespie, 1961; Cripps *et al*, 1976), and measurement of all the factors which can effect the success of an LTSF cycle is difficult, making successful cycle parameters difficult to determine and guarantee. The only reliable method of measuring the affect of all the parameters on the effectiveness of a sterilization cycle is to use a Biological Indicator (BI), to integrate all the variables and hence monitor the lethality of the process.

Biological Indicators have traditionally been prepared using bacterial spores which exhibit high resistance to the sterilizing conditions under test. The ideal characteristics for a BI for use in LTSF monitoring should therefore include high resistance to the temperatures and formaldehyde concentrations used in LTSF. However, they should also include characteristics such as ease of production off a simple chemically defined medium, linear inactivation kinetics, reproducibility between batches and also be aerobic and non-

pathogenic. It is also desirable to use a thermophile, to reduce the risk of false positives from chance environmental contamination during incubation of the tests. Until such a monitor is available LTSF cannot be utilized to the full potential, and will continue to be considered a disinfection rather than a sterilization process in such institutions as the NHS (CSC Report, 1986). The work reported on in this thesis was sponsored by the DoH as part of the ongoing research to develop a reliable Biological Indicator organism to monitor LTSF sterilization.

The first part of this work reported on the screening of 16 strains of *Bacillus stearothermophilus* sporulated on four different defined media. The growth and sporulation characteristics of the organisms were compared, and ones exhibiting acceptable characteristics were assigned batch numbers and clean spore suspensions prepared. These were then further characterised as to their ease of cleaning, Growth Index (GI), resistance to 0.5% aqueous formaldehyde at 70°C and the type of inactivation kinetics exhibited. From these results, two organisms exhibiting the best combination of characteristics were chosen for further study. The best of these two was used in all further work whilst the other was retained as a backup.

A test apparatus to investigate the effect of defined LTSF conditions upon the candidate BI organism was then developed. This involved the modification of a commercial LTSF sterilizer to allow cycle parameters to be varied and samples of the test organism introduced and removed during a cycle. This part of the investigation also involved the development of a protocol for measurement of the concentration of gaseous formaldehyde inside the chamber.

The modified LTSF sterilizer was used to investigate the effects of variations in temperature and formaldehyde concentration on the effectiveness of the LTSF process on the candidate organism. This demonstrated the linearity of kinetics and high resistance of the organism under LTSF conditions. This also confirmed the reliability of the test system.

Finally, the report by Spicher and Peters (1976; 1981), that the number of spores which survived exposure to formaldehyde could be increased by a heat treatment was investigated. The first part of this study was carried out in the aqueous inactivation conditions used in Chapter 3, as this most closely resembled those used by Spicher and Peters. The results of this investigation, that heat did induce recovery of spores which previously were incapable of forming visible colonies, warranted further investigation. The effect of heat treatment on the recovery of spores inactivated by LTSF conditions was therefore investigated.

Chapter 3 reported on the initial screening of 16 strains of *B. stearothermophilus* obtained from three culture collections. These were grown and sporulated on four defined media described by previous investigators, two solid media, one liquid and one in both solid and liquid form. (Hoxey, 1984; Steele, 1987). Of all the organisms, only *B stearothermophilus* NCIB 8224 was sporulated on only one medium, Carbon limited (C-limited), as it had already been proven unsuccessful on the other three media in a previous study (Hoxey, 1984). Of the sixteen strains, only 16 combinations of organism and medium produced batches of spores that met the selection criteria of good growth, >50% sporulation and ease of cleaning. Several of the "unsuccessful" combinations had good growth and sporulation, but had problems with clumping of the spores, and as the aim was to use no

chemical treatment such as polysorbate (Tween), these batches were not considered further.

The batches which met the criteria stated above were then exposed to inactivating conditions of 0.5% aqueous formaldehyde at 70°C. These conditions were chosen to determine the resistance characteristics of the test strains to a combination of high temperature and formaldehyde whilst still giving a range of D-values which allowed practical sampling times.

The results of these investigations (summarized in Table 3.17), demonstrate that the strains selected for aqueous inactivation studies exhibit a wide range of characteristics in GI, % sporulation and T_3 value. These variations also include the shape of the survivor curves obtained (Appendix II and Fig. 3.2), with examples of activation, shouldered, linear, sigmoidal and tailing survivor curves obtained.

The most prevalent of the types of survivor curve was the log-linear reduction, which agrees with the statement that most organisms demonstrate this type of curve (Schull *et al*, 1963). The batches with low growth indices generally produced curves of activation or shouldered type, and ones with higher GIs (>50%) appeared to show none of this heat activation. The T_3 values varied greatly, showing a range from 20 minutes for spores of DSM 2349 sporulated on DeGuzman's medium to 111 minutes for NCIB 8224 sporulated on C-limited medium. The results of this study support other reports that sporulation medium has a great affect on the characteristics of spores produced on it (Lee and Brown, 1975; Waites *et al*, 1978; Waites and Bayliss, 1980; Dadd *et al*, 1983a). Examples of this can be seen with ATCC 15952 and ATCC 12016, both of which exhibited varying characteristics when sporulated on different media. In

particular, even different forms of the same media have a great effect on resistance characteristics (e.g ATCC 12016 in liquid and on solid SSMAVIT). From these results, two strains, NCIB 8224 and ATCC 15952 were considered to exhibit the best combination of characteristics. Further work with these two organisms also demonstrated that they both exhibited reproducible characteristics (D-value and GI) both within and between batches (Tables 3.21 - 3.24).

From the data presented in Chapter 3, it can be concluded that a whole range of parameters needs to be standardized to produce a reliable BI for use in LTSF. Stating the organism and strain alone is not enough, the type and form of the sporulation medium, incubation temperature and method of harvesting and cleaning the spores must be stated. From the results reported by Chinyanganya (1989), storage conditions and time should also be specified to compensate for resistance changes during storage. These are not all the factors needed to guarantee reproducible results; as will be discussed later, recovery conditions play a great part in the results obtained from a BI. Finally, from the studies on the 16 strains of *Bacillus stearothermophilus*, it can be concluded that NCIB 8224, sporulated on C-limited medium, exhibited the most desirable combination of characteristics, with ATCC 15952 as the next most suitable choice. For this reason, NCIB 8224 was considered the primary candidate BI organism and ATCC 15952 the backup in case of unforseen problems with the other.

Chapter 4 reported on the development of a test apparatus for exposing spores of the candidate BI to controlled LTSF conditions. The basis of the apparatus was a commercial Thackray Miniclave 80 LTSF sterilizer. The modification was carried out in conjuction with

S.J.Line from the PHLS Norwich and F.W. Chinyanganya from the University of Bath. The modifications included addition of a calibrated temperature controller for the chamber heating jacket and a heating mat and controller for the chamber door. The door modification was considered necessary after condensation problems were encountered by Chinyanganya. The steam supply from the vaporiser used in the commercial machine was replaced with an electric Portaclave for studies by Chinyanganya. This was then replaced with a more reliable "Little Sister" autoclave, modified to supply good dry saturated steam at 110°C, allowing many cycles a day to be run without addition of more water. One of the most important modifications carried out to the Miniclave 80 was the addition of the formalin injection system and its associated controls and assay protocol. The 'single injection' system, used in studies carried out by Chinyanganya was further modified to the present dual syringe injection system. This allowed a "top up" amount of formaldehyde to be injected during the cycle to offset the continual loss that was demonstrated in Figure 4.9. This led to an improvement, giving constant concentration of 12 μg/ml ± 3 μg/ml (Fig. 4.10), which is as accurate as could be expected with the present controls, as a pulsed system will always produce peaks and troughs in the concentration. To monitor the effectiveness of the formaldehyde injection system, an assay system was chosen and validated and a protocol for sampling and assaying formaldehyde concentration developed. After all the modifications were complete, this apparatus allowed the exposure of spores to defined LTSF conditions, between 63°C and 83°C ±1°C and formaldehyde concentrations of 3 μg/ml ± 0.75 μg/ml to 17 μg/ml ± 3 μg/ml. This allowed the production of reproducible LTSF survivor curves as shown in Figure 4.12.

With the development of the test apparatus, it was finally possible to test the candidate BI organism under the conditions it would be used to monitor, that is LTSF as opposed to aqueous formaldehyde. Chapter 5 reported on the effect of both temperature and formaldehyde concentration on the survival of spores of *B. stearothermophilus* NCIB 8224 sporulated on C-limited medium when exposed to LTSF conditions. It was found that temperature changes of between 63°C and 83°C in 5°C increments had no discernible effect on the inactivation rate of LTSF at a formaldehyde concentration of 12 µg/ml ± 3 µg/ml. This is what would be expected from the results reported by Hoxey (1984), that temperatures over a similar range had no effect on inactivation of spores of various *Bacillus* spp. by LTSF. The next section of this chapter reported on the effect of formaldehyde concentration on the rate of inactivation of spores. A plot of formaldehyde concentration against inactivation rate constant (k) (Figure 5.11) demonstrates clearly that there is a linear relationship of increasing inactivation rate with increasing concentration up to a concentration of approximately 12 µg/ml formaldehyde. Any increase in formaldehyde concentration beyond this point leads to no further increase in the rate of reaction. This suggests that some saturation point has been reached. This could mean that all the sites of action of the formaldehyde, for example alkylation sites, are occupied, and hence an increase in formaldehyde concentration will not lead to an increase in inactivation rate. Another method of expressing the effect of concentration on inactivation rate of cidal agents is the concentration exponent (n). When this value was calculated using the three points which showed a linear relationship, a value of 1.4 was obtained for n. This value is of the same magnitude as the figure of 1 quoted by various workers

for aqueous formaldehyde sterilization (Russell, 1987; Hugo and Denyer, 1987). This figure is thought to relate to the inactivation mechanism of the sterilization process. This therefore implies that both aqueous and gaseous formaldehyde may inactivate spores by the same or a similar mechanism.

Finally, Chapter 6 reported on investigations into the effect of recovery conditions on survival of spores inactivated by formaldehyde. In particular, the effect of heat treatment on spores exposed to formaldehyde reported by Spicher and Peters (1976 and 1981) and Gorman *et al* (1983) was studied. The original system described by Spicher and Peters was exposure of *B. subtilis* to 15% aqueous formaldehyde at 20°C. The investigations reported here were studied at the more relevant conditions of 70°C with 0.5% aqueous formaldehyde, those used to screen for potential BI organisms for LTSF. The results of these experiments were conclusive that after exposure to aqueous formaldehyde the number of survivors could be increased by a heat treatment of 85°C to 97°C prior to incubation, compared to those recovered by incubation at 55°C alone. The optimum conditions appeared to be 90°C heat treatment for 40-60 minutes just prior to plating. A treatment at 85°C took a longer time to attain a lower maximum than 90°C, whilst 97°C exhibited signs of inactivating the spores after a brief reactivation. After optimum treatment, the largest increase in survivors obtained was 1.5 log cycles, and this was considered worth further investigation. The effect of extended incubation of spores, which had been exposed to 0.5% aqueous formaldehyde, on nutrient agar at 55°C for up to 14 days was investigated. This was found not to give as high a recovery as that obtained by heat treatment. This confirms the results of Ortenzio, 1966, who demonstrated that extended incubation could not reproduce

the effect of heat treatment on recovery of spores.

Once the development of the LTSF apparatus was complete, it was possible to investigate the effect of heat treatment on spores inactivated by LTSF. These studies demonstrated that the phenomenon does not just occur in aqueous systems but also in gaseous ones. It also demonstrated that the treatment was more effective on spores exposed to LTSF than those exposed to aqueous formaldehyde. The percentage recovery of spores neared 100%, particularly for spores exposed to LTSF at higher temperatures. To compare the effects of heat treatment on spores exposed to LTSF at different temperatures, the ratio of k values of the survivor curves for treated an untreated spores was calculated. These ratios clearly demonstrate that heat treatment has a much greater effect on spores exposed to LTSF at 78°C and 83°C (giving 15- and 70-fold differences in k value), than on spores exposed to LTSF at lower temperatures. It is suspected that the final few percent of spores that were not recovered could have been physically lost as discussed in Section 5.3. If this were found to be not so, then it is possible that even these may be recovered by optimisation of the heat treatment for LTSF or a combination of heat treatment with one of the alternative methods discussed in 6.3. If this was proved to be possible, then it could be demonstrated that formaldehyde has no immediate permanent sporocidal activity at all, but is either sporocidal later in recovery or is inhibitory to germination. This is not a new idea, it has been suggested that formaldehyde is likely to act on nucleic acids as its lethal site (Trujillo and David, 1972). DNA is not accessable to formaldehyde attack except when the double strands open up during synthesis (Chattoraj, 1970; Kozlov and Debabov, 1972). It is therefore more likely that formaldehyde binds to the thiol groups within the spore

coat (Dadd and Daley, 1982) and acts as a germination inhibitor. The lethal effect could then occur when germination takes place, and the DNA becomes accessible. One way of determining if the lethal effect does occur during germination would be to force the spores to germinate immediately after inactivation but prior to heat treatment. If the spores did not germinate (remained phase bright) then germination inhibition would be indicated. If the spores did germinate (changed to phase dark), and then heat treatment did not increase the number of survivors recovered, then the lethal effect must occur during or just after germination. If however, the heat treatment still recovered an increased number of survivors, then it would mean no permanent inactivation was caused by formaldehyde. As reported in 6.3 however, stimulation of germination of spores that had not been exposed to formaldehyde, by a germination mixture described by Russell (1989) proved unsuccessful. This result appears to confirm the statement that spores of *B. stearothermophilus* can not be chemically germinated but require a heat shock treatment at above 100°C (Keynan *et al*, 1965). If this is not true however, then further investigation using other germination stimulating mixtures which have been described might prove successful; some of these include O-carbamyl-D-serine and L-Alanine analogues (Irie *et al*, 1984; Titball and Manchee, 1987; Kanda *et al*, 1988)

The results of the heat treatment investigations have implications for the use of formaldehyde as a sterilant. Other spore-forming organisms could well exhibit this recovery after heat treatment. It is therefore possible that pathogenic spore-formers could survive to be recovered at a later date, or possibly recover in the steam flush elution (itself a type of heat treatment) used in many commercial LTSF sterilizers. This possibly could account for the variation of

success reported for LTSF. Of equal importance is the fact that the recovery conditions of the BI should be standardized to prevent differences in those conditions causing discrepancies in results (though in the case of heat treatment it would be a safe, false positive, result). This standardisation should extend itself to the designing/choosing of a defined recovery medium, as composition of recovery media has been shown to have an effect on recovery (Ernst, 1968; Briggs and Yazdany, 1970; Roberts, 1970; Mallidis and Scholefield, 1986).

From the results reported in this work it is possible to recommend that *B. stearothermophilus* NCIB 8224 sporulated on C-limited medium is a good choice for a BI for LTSF. This organism demonstrated many of the desirable attributes of an ideal BI. These include ease of production on simple defined sporulation medium and high resistance to LTSF conditions with reproducible linear inactivation kinetics. The Growth Index is not as high as could be desired, but as it is reproducible, and the organism does not exhibit heat activation, it is acceptable.

7.2 SUGGESTIONS FOR FURTHER WORK

For further development of the protocol for monitoring LTSF cycles, it will be necessary to develop a defined recovery medium. This, like the sporulation medium, should be as simple as possible for economic reasons. It should give a high and reproducible recovery without effecting the linearity of the survival curve obtained.

Development of the LTSF test apparatus should continue. The addition of a more reliable and automatic formaldehyde injection/monitoring unit is the most important. This could include

a device such as the piezoelectric crystal assay system as described in 4.3. This should be connected to a computer which can control adjustment of formaldehyde injection automatically hence giving more defined LTSF conditions.

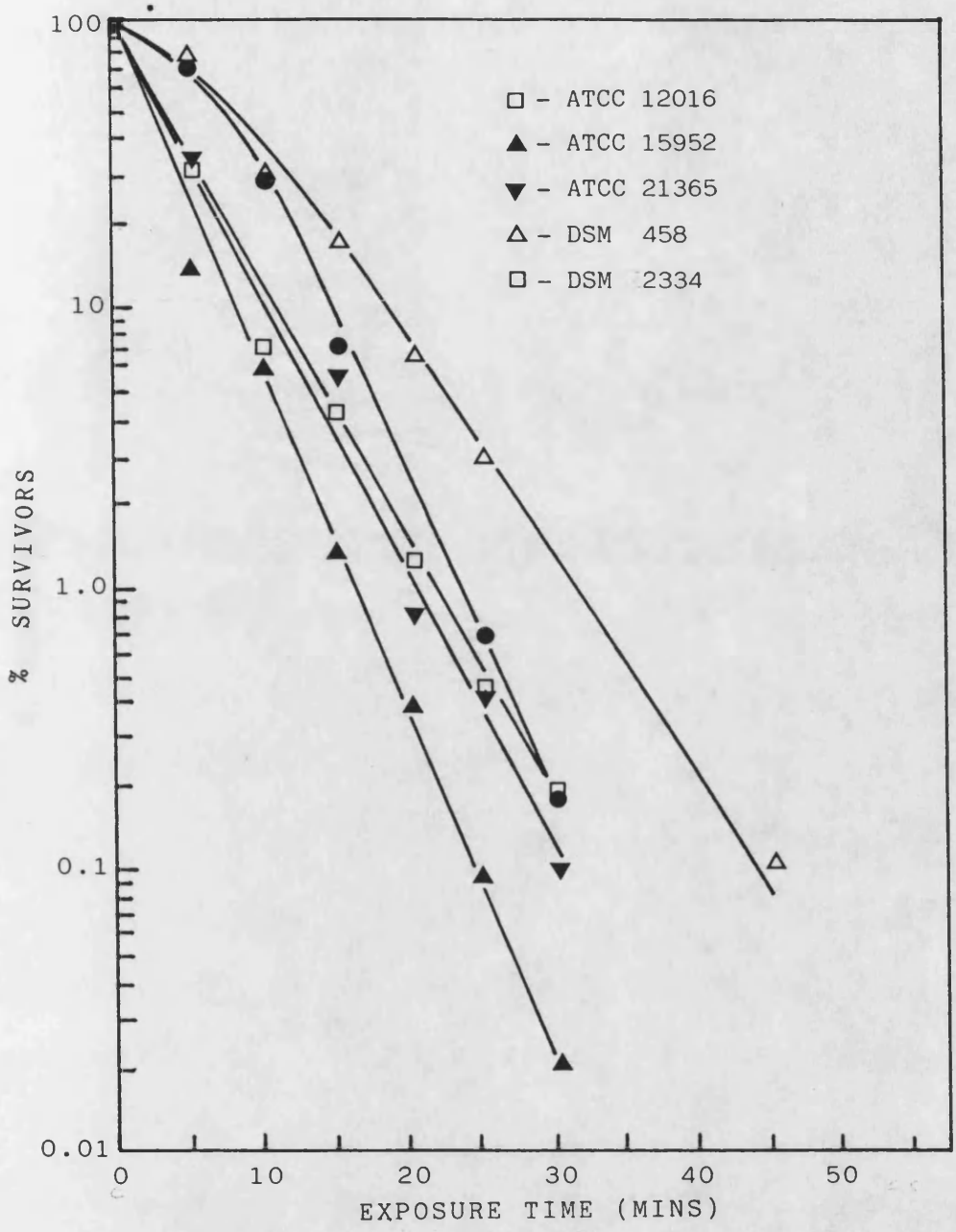
Of great importance, the mechanism of action of formaldehyde on bacteria and spores should be investigated. In particular, the question of whether or not formaldehyde is sporocidal should be addressed. This should include further investigation of heat-induced revival of spores. Firstly, the heat treatment conditions should be optimised for use with spores which have been exposed to LTSF. Secondly, it should be ascertained whether the magnitude of the increase in survivors is dependant on the concentration of formaldehyde the spores are exposed to. Another area of investigation would be to try alternative methods of enhanced recovery (e.g. abrasion and lysozyme) both singly and in combination with heat treatment, to attempt to recover the final few percent of spores not recovered by heat treatment. The use of other chemical germinants should be attempted, to determine at which point heat treatment becomes ineffective and hence pinpoint the mechanism of lethality of formaldehyde. Next, it should be determined whether spores of potential pathogens (e.g. *Clostridium* spp.) exhibit this recovery. Finally, it should be demonstrated whether or not other conditions, to which LTSF sterilized articles may be subjected, could cause similar recovery. The mechanism of formaldehyde action could be investigated using radio-labelled formaldehyde to identify the site of action.

APPENDICES

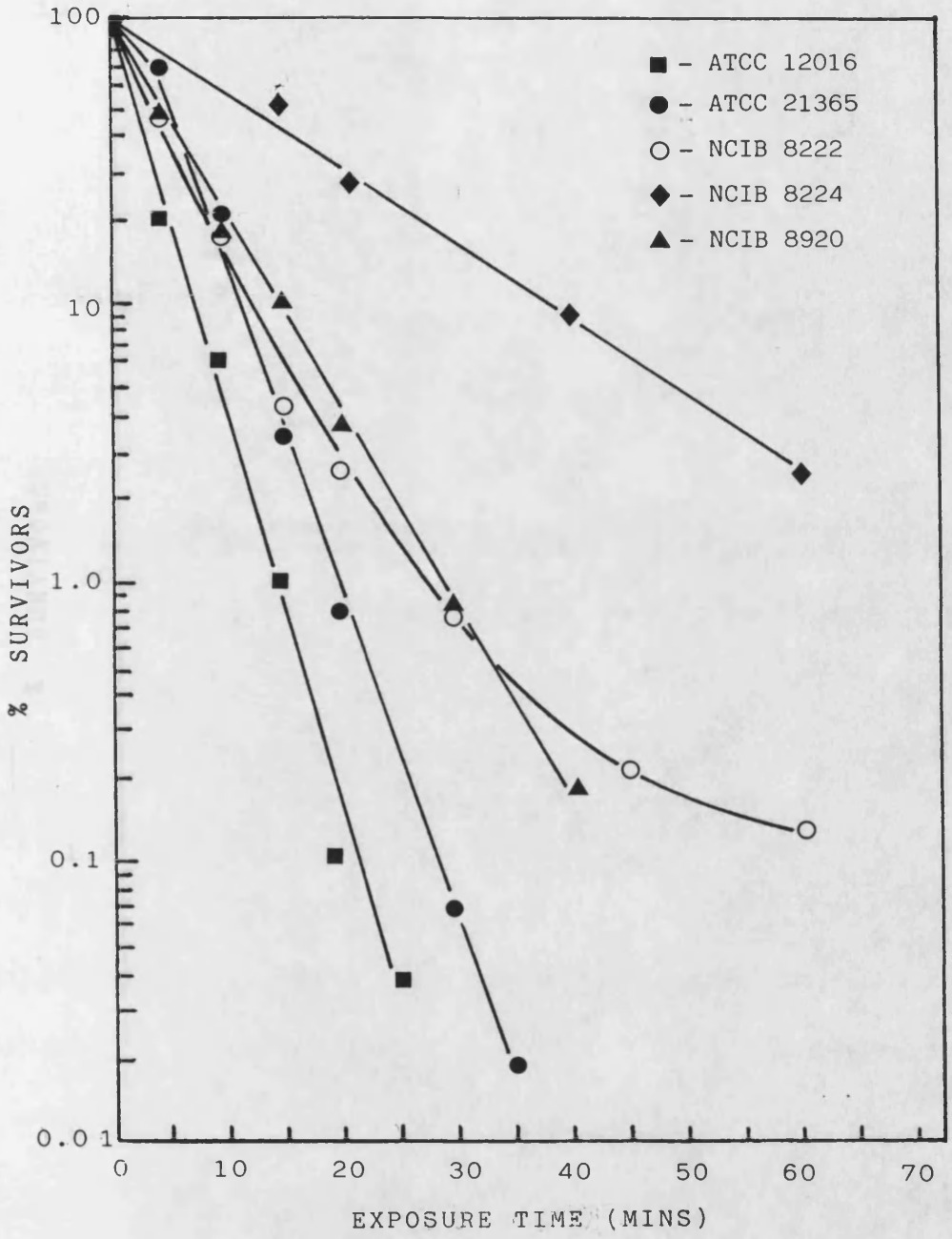
APPENDIX I

A1.1. Gravimetric Determination of the Accuracy and Reproducibility of Gilson Pipettes at Volume settings Relevant to this Study.

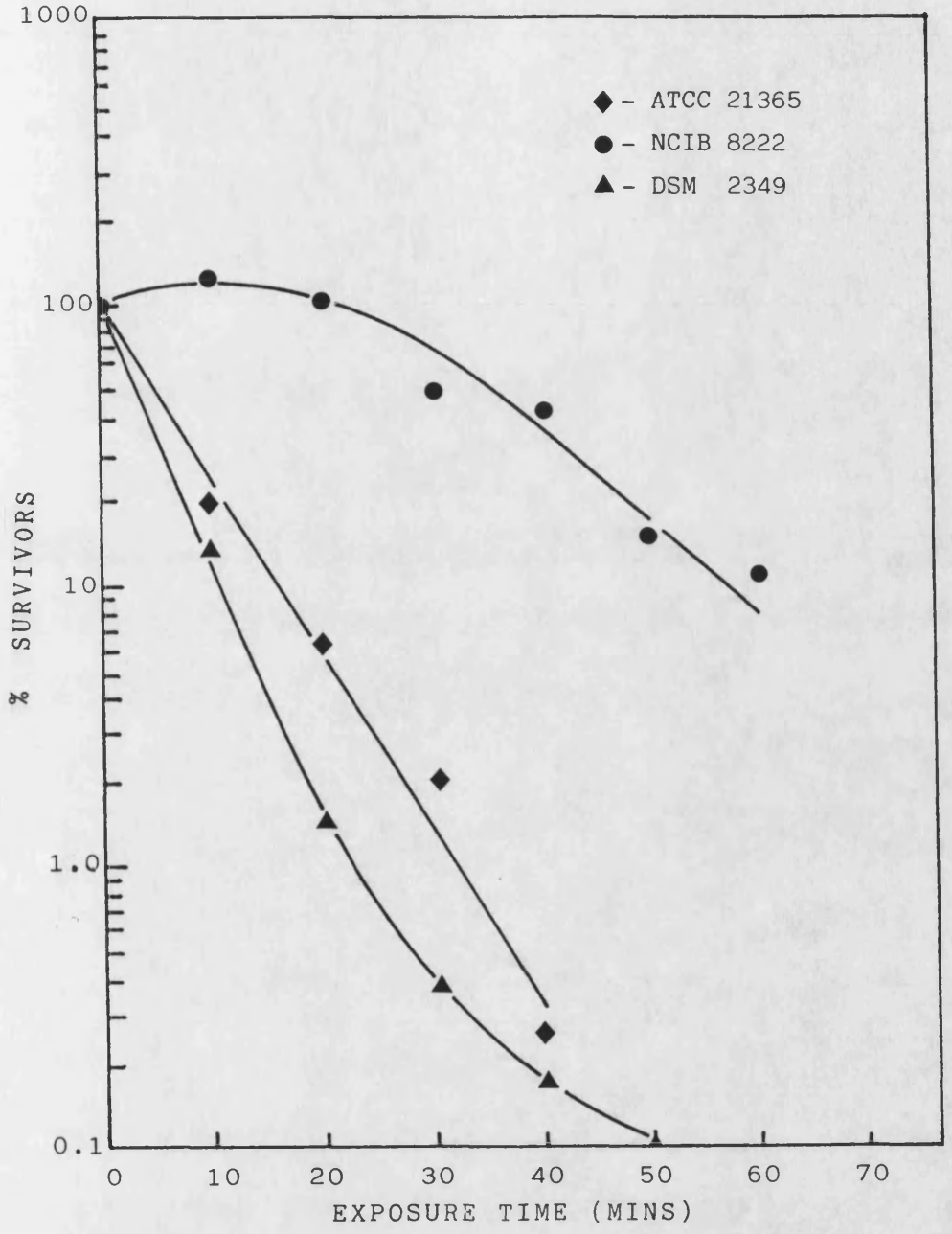
Pipette Model	P200	P1000	P1000	P5000
Volume set	(20µl)	(1 ml)	(0.2 ml)	(4.5 ml)
Weight of water from Replicate volume Measurements (g)	0.0221	0.9980	0.2028	4.5123
	0.0199	0.9940	0.1992	4.5101
	0.0207	0.9973	0.2016	4.5220
	0.0207	0.9996	0.1999	4.5192
	0.0202	0.9902	0.2023	4.5207
	0.0209	0.9993	0.2034	4.5996
	0.0199	0.9862	0.2004	4.5320
	0.0209	0.9871	0.2050	4.5209
	0.0205	0.9856	0.2019	4.5311
	0.0209	0.9979	0.2009	4.5270
Mean	0.0206	0.9935	0.2017	4.5215
Standard Deviation	0.000636	0.0057	0.00174	0.00713
Coefficient of Variation	3.1%	0.57%	0.86%	0.71%
Theoretical Mass at 25°C	0.0199	0.99707	0.1994	4.4868
Error	3.5%	0.36%	1.15%	0.77%



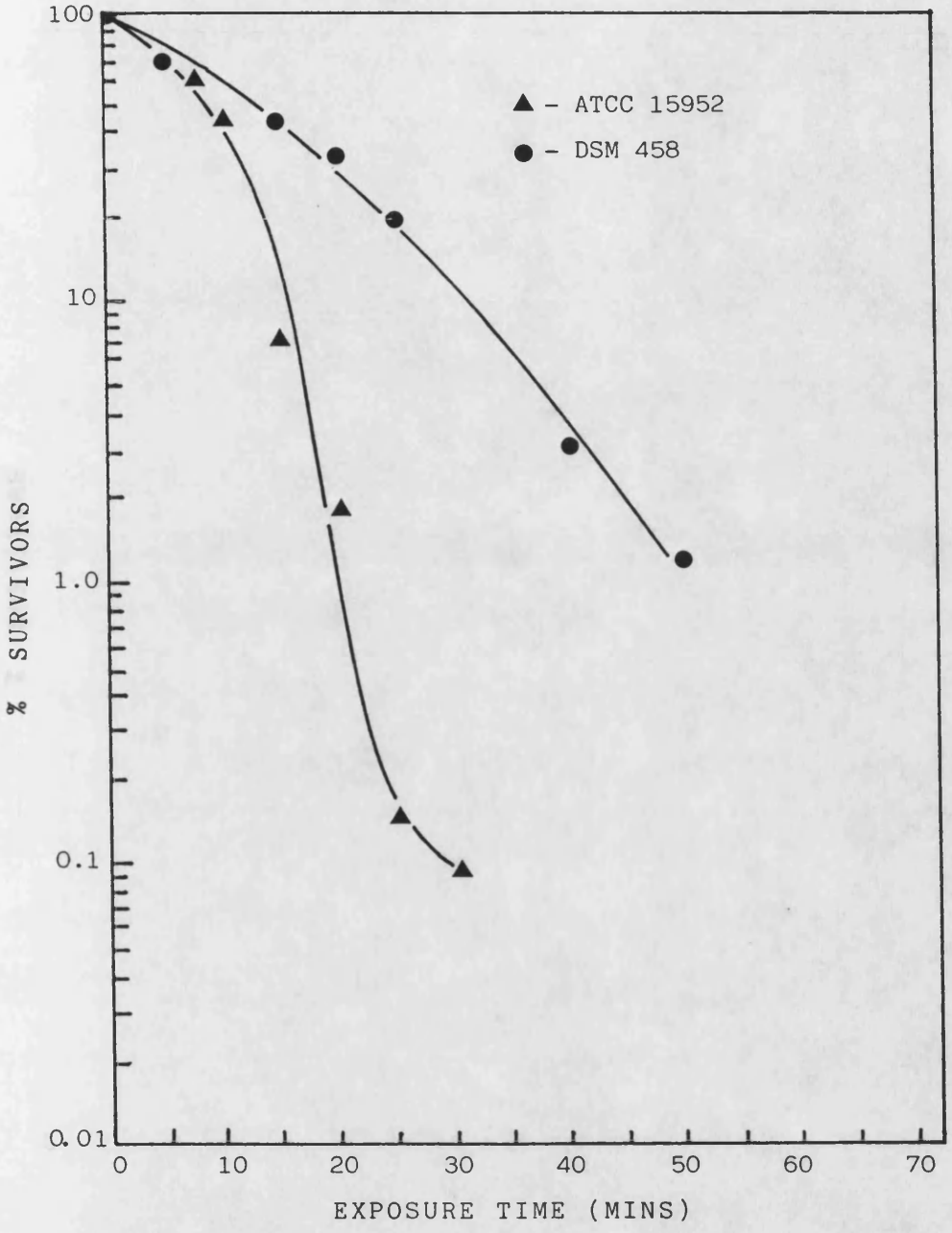
A2.1 Survivor Curves for Spores of B. stearothermophilus Produced on Solid SSMAVIT Medium When Exposed to 0.5% Aqueous Formaldehyde at 70°C



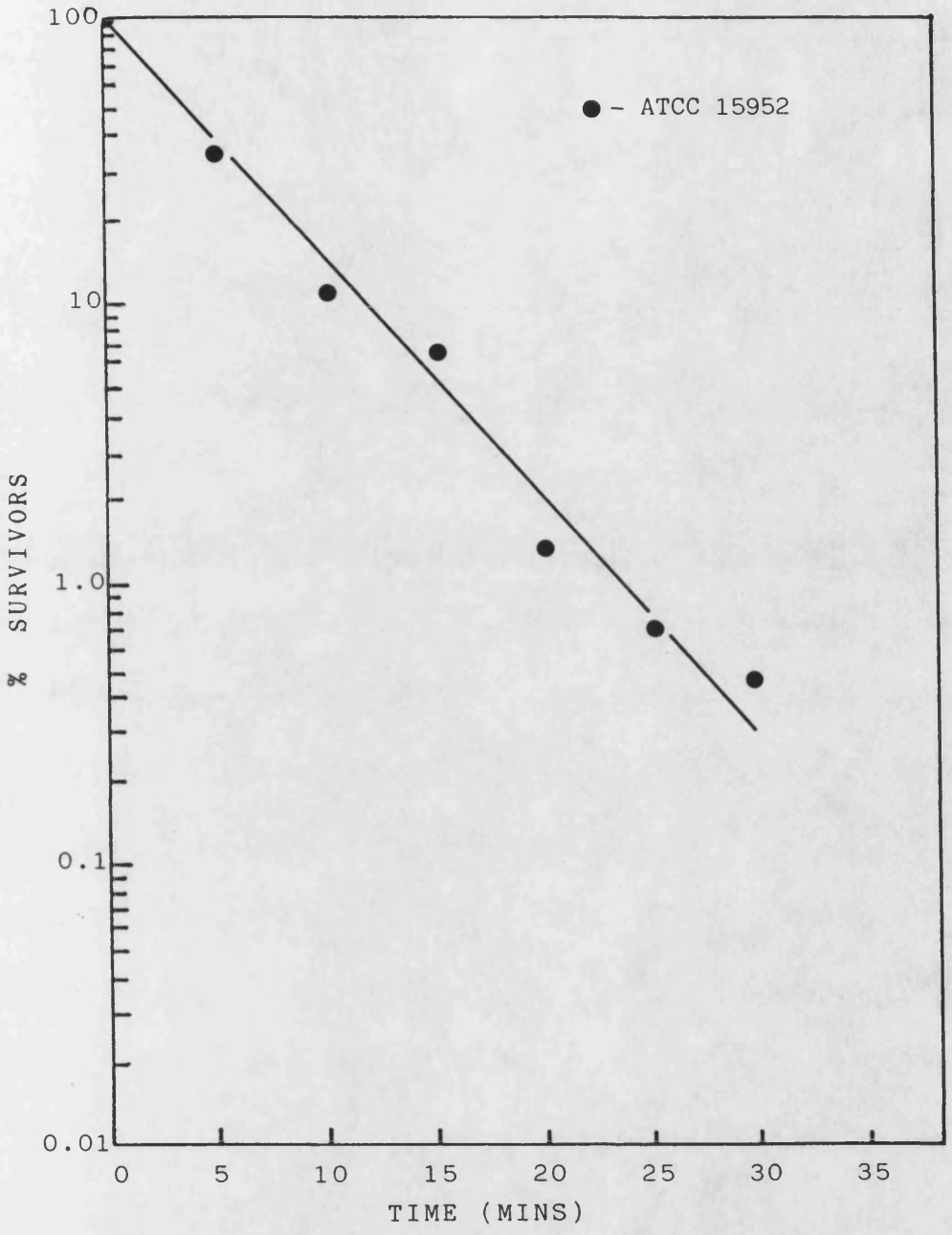
A2.2 Survivor Curves for Spores of B.stearothermophilus Produced on Carbon Limited Medium When Exposed to 0.5% Aqueous Formaldehyde at 70°C



A2.3 Survivor Curves for Spores of B. stearothermophilus Produced on De Guzman's Medium When Exposed to 0.5% Aqueous Formaldehyde at 70°C



A2.4 Survivor Curves for Spores of B. stearothermophilus Produced on Liquid SSMAVIT Medium When Exposed to 0.5% Aqueous Formaldehyde at 70°C



A2.5 Survivor Curve for Spores of B. stearothermophilus Produced on Anderson's Medium When Exposed to 0.5% Aqueous Formaldehyde at 70°C

APPENDIX III

STATISTICAL ANALYSIS

A3.1 Standard Error of the Mean

When an exprimental measurement is repeated under apparently identical conditions, and varying results are obtained, the extent of scatter around their mean value gives a measure of the random error invoved in the experiment. The mean, M, of a range of experimental points x_1, x_2, x_3... x_N can be calculated as :

$$M = (x)/N$$

The variance, V, of this mean is expressed as :

$$V = \{(x-M)^2\}/N$$

and the standard deviation, s, of the mean is :

$$s = \sqrt{V}$$

To graphically represent the random error obtained in an experiment, the satndard error, SE, is used. This is then graphically represented m ± SE, with the SE shown as a bar. The SE is calculated from the standard deviation as :

$$SE = s/\sqrt{N}$$

A3.2 Least Squuares Regression Analysis

When a linear relationship is assumed to exist, it is usual to fit a straight line by least squares regression analysis. This will give the best fitting relation between two quantities, one liable to chance error known as the dependant variable (y), which is normally distributed around the mean with a variance V. The other is assumed to be measured without error known as the independant variable (x).

The method of least squares obtains estimates of c and m in the equation $y = mx + c$ such that the sum of the squares of the deviations of the observations y_i from their mean is a minimum.

These values are :-

$$m = \frac{n\Sigma x_i y_i - \Sigma x_1 \Sigma y_1}{n\Sigma x_1^2 - [\Sigma x_1]^2}$$

$$= \frac{\Sigma (x_i - \bar{X})\ (y_i - \bar{y})}{\Sigma (x_i - x)^2}$$

$$c = \frac{\Sigma y_i - m\Sigma x_i}{n}$$

$$= \bar{y} - m\bar{x}$$

where n is the number of points on the line

Variance of the Slope (m)

This is termed s_m^2 and is given by the equation :

$$s_m^2 = \frac{\sigma e^2}{\Sigma(x_i-\bar{x})^2}$$

Where 2_e is the residual varianve of the dependant variable y and may be obtained by dividing the residual sum of squares D^2 by (n-2) where :

$$\Sigma D^2 = \Sigma(y_i-\bar{y})^2 - \left[\frac{\Sigma(x_i-\bar{x})\ (y_i-\bar{y})^2}{\Sigma(x_i-\bar{x})^2}\right]$$

Residual sum of squares (n-2) degrees of freedom	Total sum of squares (n-1) degrees of freedom	Sum of squares due to regression 1 degree of freedom

The denominator (n-2) shows that two degrees of freedom have been lost as both the slope and intercept were calculated from the data. The standard deviation of the slope is given by the square root of the variance.

Variance of the Intercept (c)

This is termed : $s^2c = \frac{\Sigma x_i^2 \sigma e^2}{n\Sigma(x_i - \bar{x})^2}$

where $\sigma^2 e = \frac{\Sigma D^2}{(n-2)}$

The standard deviation of the intercept is given by the square root of the variance.

A3.3 To Demonstrate the Equality of two Estimates of a Parameter. (Student's t-test)

The equality of estimates of a parameter, p, p_1 and p_2, with variances s_1^2 and s_2^2 respectively is assessed by :

$$t = \frac{p_1 - p_2}{(s^2_{\ 1} + s2_2)^{1/2}}$$

The value of t is compared with tabulated values with $n_1 + n_2 - 4$ degrees of freedom, where n_1 and n_2 are the numbers of observations used in the estimation of p_1 and p_2 respectively. If the value of the experimental t (t_{exp}) does not exceed the tabulated t value (t_{tab}) at the 5% level (p=0.05) then there is assumed to be no significant difference at this level of probability.

A3.4 To Demonstrate the Equality of Two Means of a Parameter (student's t-test)

The equality of means x_1 and x_2, with variances $s^{2n}T_1$ and s^2_2 respectively, can be assessed as follows :

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s(1/n_1 + 1/n_2)^{1/2}}$$

where n_1 and n_2 are the number of observations used to estimate x_1 and x_2. s represents the total standard deviation given by :

$$s = \frac{(n_1-1)s_1^2 + P(n_2-1)s_2^2}{(n_1 + n_2) - 2}^{1/2}$$

The value ue of t, t(exp) is compared with the tabulated values with $n_1 + n_2 - 2$ degrees of freedom. The conclusions are as for the t-test in A2.3

A3.5 To demonstrate the Equality of More than Two Means of a Parameter (Analysis of Variance).

If more than two means of a number of experimental observations are to be compared, an F-ratio is calculated using the Analysis of Variance. The following values must be calculated :

A) The uncorrected sum of Squares

$$= \sum_{i=1}^{k} \sum_{j=1}^{n} x_{ij}^2$$

where k is the number of groups, in is the number of observations in each group and x^2 is the square of the observation or measurement.

B) Mean Sum of Squares of Group Totals

$$= \sum_{i=1}^{k} \frac{\left(\sum_{j=1}^{n} x_{ij}\right)^2}{n_1}$$

C) Mean Sum of Squares of Observations

$$= \frac{\left[\sum_{i=1}^{k} \sum_{j=1}^{n} xij\right]^2}{N}$$

where N is the total number of observations

An Analysis of Variance table is now constructed :

Source	Sum of Squares	Degrees of Fredom	Variance
Between Groups	B-C	k-1	$\frac{B-C}{k-1} = s_2^2$
Within Groups	A-B	n-1	$\frac{A-B}{N-k} = s_1^2$
Residual	By subtraction	(k-1)(n-1)	
Total	A-C	N-1	$\frac{A-C}{N-1}$

The F ratio, s_2^2/s_1^2 will come from an F distribution, and if there is no significant difference between the variation within and between groups, then the F-value will be less than that tabulated at p = 0.05 (5%) for k-1 and N-K degrees of freedom.

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